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Promoteur : Pr Didier Serteyn

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Promoteur : Pr Patsy Renard

COTUTELLE

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Lola Dechêne

THESE PRESENTEE EN VUE DE L'OBTENTION DU GRADE DE DOCTEUR EN SCIENCES VETERINAIRES ET EN SCIENCES

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la grandeur de ton implication à mes côtés durant ces 4 années et il n'y aura pas assez de mercis pour cela.

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Abbreviations

ADAMTs	A Disintegrin And Metalloproteinase with Thrombospondin motifs
AFMPS	Agence Fédérale des Médicaments et des Produits de Santé
BM	Bone marrow
BSA	Bovine serum albumin
CID	Collision-induced dissociation
COMP	Cartilage oligometric matrix protein release
COX	Cyclooxygenase
DMEM	Dulbecco's Modified Eagle Medium
DTT	Dithiothreitol
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EV	Extracellular vesicles
ER	Endoplasmic reticulum
FASP	Filtered-aided sample preparation
FBS	Fetal Bovine Serum
FCCP	Trifluoromethoxy carbonylcyanide phenylhydrazone
FCR	Flux control ratio
FDR	False Discovery Rate
GAG	Glycosaminoglycan
GAPDH	Glyceraldhehyde-3-phosphate dehydrogenase
HA	Hyaluronic acid
HPβCD	Hydroxypropyl-beta-cyclodextrin
IAA	Iodoacetamide
IGFBP	Insulin growth factor binding protein
IL	Interleukin
INF-γ	Interferon gamma
IRAP	Interleukin-1 Receptor Antagonist Protein
mdMSCs	Muscle-derived mesenchymal stem cells
MHC	Major Histocompatibility Complex
MMPs	Matrix metalloproteinases
MS	Mass Spectrometry
MSCs	Mesenchymal Stem Cells
NFKB	Nuclear factor kappa B
NO_2^-	Nitrite

O/N	Overnight
OA	Osteoarthritis
OCR	Oxygen consumption ratio
PFA	Paraformaldehyde
PASEF	Parallel accumulation serial fragmentation
PB	Peripheral blood
PBS	Phosphate-Buffered Saline
PCM	Pericellular matrix
PGE2	Prostaglandin E2
PRP	Platelet-rich plasma
PTM	Post-translational modifications
PVDF	polyvinylidene fluoride
RAO	Recurrent airway obstruction
RPM	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
SERPIN	Serine proteinase inhibitors
SILAC	Stable isotope labeling by amino acids in cell culture
SLRP	Small leucine-rich proteoglycans
SUIT	Substrate-uncoupler-inhibitor titration
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TGF	Transforming growth factor
TIMPs	Tissue inhibitors of metalloproteinases
TNF-α	Tumor necrosis factor alpha

Abstract

Abstract

Osteoarthritis is a high prevalent joint degenerative disease for which therapeutic treatments are limited or invasive. Cellular therapy based on mesenchymal stem cells is therefore seen as a promising approach for this disease. This research project works with equine mesenchymal stem cells harvested in muscle, an easy access site, with a minimally invasive process.

As curcumin is described to improve the osteoarthritis pathology by decreasing the pain and the stiffness of the joint, the goal of this project was to study the combination of both muscle-derived mesenchymal stem cells (mdMSCs) and a hydrosoluble form of curcumin called NDS27.

We have first developed an *in vitro* model of osteoarthritis disease in order to evaluate the potential clinical effect of this innovative product. This model is focus on cartilage (*ex vivo* explant) and its response in pro-inflammatory (TNF-α and IL-1β) culture conditions.

Then, as regenerative potential of MSCs is mainly conferred by paracrine function, we have characterized the secretome of mdMSCs in this *in vitro* model of OA (explant of cartilage with TNF-α and IL-1β). Mass spectrometry analyses identified several actors secreted by mdMSCs. According to the dual function of these secreted proteins, implicated both in catabolic and anabolic cartilage functions, we performed functional tests consisting of studying the effect of mdMSCs cultured with explants of cartilage in pro-inflammatory model. Results showed that mdMSCs decreased the glycosaminoglycan release from plugs of cartilage in this model, supporting the anti-catabolic effect of mdMSCs in these conditions, potentially explained by the increase of secreted decorin and the decreased abundance and activity of MMP3.

Finally, we have demonstrated that NDS27 is able to penetrate into mdMSCs and localizes into mitochondria, at least partially. Optimizations between conditions leading to maximal incorporation of NDS27, regenerative properties and constraints of industrial partner (to fit with good manufacturing practice) must now be done.

Résumé

L'arthrose est une maladie dégénérative articulaire touchant un grand nombre de personnes et pour laquelle, peu d'options thérapeutiques existent, hormis les traitements traditionnels ou invasifs. Les thérapies cellulaires à base de cellules souches mésenchymateuses sont vues comme des traitements prometteurs en raison de leurs capacités régénératrices. Ce travail de recherche a étudié des cellules souches mésenchymateuses récoltées dans les muscles de chevaux par une technique peu invasive et facile à réaliser.

La curcumine est un polyphénol largement décrit pour ses effets bénéfiques sur les articulations arthrosiques en réduisant notamment la douleur et la raideur provoquées par cette maladie. L'objectif de ce travail est d'étudier le potentiel thérapeutique de cellules souches mésenchymateuses d'origine musculaire (mdMSCs) traitées avec une molécule de curcumine soluble appelée NDS27.

Nous avons tout d'abord développé un modèle *in vitro* d'arthrose afin d'étudier le potentiel thérapeutique du produit étudié. Ce modèle consiste à placer en culture un prélèvement *ex-vivo* de cartilage avec un milieu pro-inflammatoire constitué de TNF-α and IL-1β.

Ensuite, comme les propriétés régénératives des cellules souches mésenchymateuses sont majoritairement attribuées à la fonction paracrine de ces cellules, nous avons caractérisé le sécrétome des mdMSCs dans ce modèle *in vitro* d'arthrose (constitué d'un explant de cartilage et des cytokines pro-inflammatoires TNF-α and IL-1β). Les analyses par spectrométrie de masse nous ont permis d'identifier des protéines sécrétées par les mdMSCs dans ces conditions. L'ensemble de ces protéines étant décrites pour exercer des fonctions anaboliques et cataboliques, nous avons réalisé un test fonctionnel pour évaluer l'effet des mdMSCs dans ce modèle. Nous avons pu montrer l'effet anti-catabolique des mdMSCs : la libération de glycosaminoglycans des explants de cartilage en milieu pro-inflammatoire était diminuée lorsque ces explants étaient cultivés en présence de mdMSCs. Cet effet est partiellement expliqué par l'augmentation de la sécrétion de la decorin et la diminution de l'abondance ainsi que l'activité de l'enzyme MMP3.

Enfin, nous avons démontré que le NDS27 était internalisé dans les mdMSCs et partiellement localisé au niveau des mitochondries. Le potentiel thérapeutique de ce produit devra encore être démontré après optimisation des conditions de préparation du produit permettant une incorporation maximale du NDS27, favorisant les propriétés régénératives du produit et respectant les contraintes de fabrication auxquelles doit répondre le partenaire industriel.

Context of this thesis

1. Context of this thesis

This thesis is part of the Curstem project which was supported by the grant WALInnov program from the Wallonie recherche SPW, convention number 1610151. This project involves several partners including another PhD program (Margaux Colin, ULB) whose research is complementary to the results presented in this manuscript. The goal of this project was to develop an innovative cell therapy product for horse osteoarthritis (OA) disease including muscle-derived mesenchymal stem cells (mdMSCs) treated with a hydrosoluble form of curcumin called NDS27.

This work includes first an introduction on mesenchymal stem cells (MSCs) and OA based on literature. Then, results are presented in 3 parts. Each part includes a brief review of literature, the material and methods, the results, the discussion and conclusions. These parts are:

- Development of the *in vitro* OA model used
- Characterization of the mdMSCs secretome
- Potential of mdMSCs use as drug-carrier for NDS27 in the context of OA disease

Finally, a general conclusion will close this research work.

Introduction

2. Introduction

2.1. Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are non-hematopoietic multipotent stem cells which were first described in 1970 (Friedenstein, Chailakhjan, & Lalykina, 1970). Physiological functions of these adult stem cells are maintenance of body homeostasis and tissue repair (Vizoso et al., 2017). In 2006, the International Society for Cellular Therapy defined minimal criteria to characterize human MSCs: plastic-adherent in cell culture, expression of CD105, CD90, CD73 and lack expression of CD45, CD34, CD19, CD14 and HLA-DR surface molecules. In addition, a last criteria concerned the MSC's *in vitro* differentiation potential to osteoblasts, adipocytes and chondroblasts (Dominici et al., 2006).

MSCs have been of increasing interest over the last years because they display several key properties for cell therapy, either for regenerative medicine or in immune diseases (N. Li & Hua, 2017). Indeed, MSCs are easy to isolate, display a large differentiation potential, and have a strong self-renewal capacity, a key feature to obtain the large number of cells required for cell therapy (Naji et al., 2019). In addition, MSCs expression of major histocompatibility complex (MHC) is low, which explains minor immunological rejection of the organism towards these cells (Harrell, Markovic, et al., 2019). Moreover, migratory and homing abilities into regions of injury are two described properties of MSCs which confer MSCs an interesting therapeutic potential (Driscoll & Patel, 2019). This rely partly on the release of proteolytic enzymes by MSCs that explains their capacity for diapedesis across the endothelium (De Becker & Van Riet, 2016). Finally, the absence of ethical controversy about MSCs contributes to their widespread use (Mocchi et al., 2020).

2.1.1. Sources of mesenchymal stem cells

The main tissue sources of MSCs are primarily the umbilical cord for neonatal tissue and bone marrow (BM) or adipose tissue for adult tissues (Hass, Kasper, Böhm, & Jacobs, 2011). However, these cells can be harvested from many other vascularized tissues like peripheral blood (PB), lung, synovial fluids, periodontal ligaments or muscles (Samsonraj et al., 2017). Skeletal muscle has the advantage to represent approximately 35% of human body mass and to be easily accessible (Ceusters et al., 2017). MSCs source is important to consider because differentiation potentials and biological features may vary depending on sampled tissue (Sacchetti et al., 2016). Neonatal tissues are of utmost interest as the MSCs yield decreases as the age of the donor increases (Kern, Eichler, Stoeve, Klüter, & Bieback, 2006).

Skeletal muscle hosts different populations of stem cells that maintain tissue homeostasis (Judson, Zhang, & Rossi, 2013). The high healing capacity of muscle suggests that it is a tissue composed of cells able to participate in tissue regeneration. mdMSCs are able to differentiate into osteoblasts,

adipocytes and chondrocytes *in vitro* and exhibit the same trophic properties than MSCs harvested from BM (Jackson et al., 2010). mdMSCs' regeneration can be altered in disease and lead to muscle fibrosis, fatty tissue accumulation and deposition of extracellular matrix (ECM) proteins (Judson et al., 2013). Satellite cells, and quiescent myoblast precursors are also muscle-specific stem cells but their differentiation potential is limited (they differentiate only in myogenic cells) compared to mdMSCs (Jackson et al., 2010). mdMSCs is described to be an attractive source for medical therapy according its relative ease of isolation and purification (Usas & Huard, 2007). A minimally invasive process based on muscular micro biopsy allowing to harvest a high yield of MSCs has been developed in horses and meets the above mentioned criteria (Ceusters et al., 2017).

2.1.2. Potential effects of MSCs

Whether the therapeutic effects of MSCs is mainly due to their differentiation properties or to their paracrine properties was a matter of debate for years. Nowadays, it is largely admitted that the principal therapeutic mechanism of mesenchymal stem cells is the paracrine function and not the engrafting and differentiating properties because the survival of cells is short (Vizoso et al., 2017). Moreover, the number of differentiated cells identified after injection is too small to explain a therapeutic effect (Ferreira et al., 2018).

However, the extent of the immunomodulatory effect of MSCs depends on several parameters. First, the immunomodulatory properties of MSCs are dependent on the inflammatory level in the microenvironment (N. Li & Hua, 2017). Pro-inflammatory stimuli lead especially the secretion of immunosuppressive molecules like TGF-β (Kim & Cho, 2016), which is also an actor inducing MSCs homing (H. Liu, Li, Zhang, & Li, 2018). These stimuli enhance the secretion of anti-inflammatory proteins (such as heme oxygenase) which could favor the anti-inflammatory phenotype of macrophages (M2) (Kim & Cho, 2016; Ragni et al., 2020). Therefore, priming MSCs with cytokines could be a strategy used to increase their trophic factor for therapies (Redondo-Castro et al., 2017).
Second, the routes for administration are important to consider because this step influences cell activity and survival in particular depending on needle types, aspiration and re-injection of cells. Routes are selected depending on pathologies and risks: thrombosis is the major risk of intra-arterial route injections (Sole et al., 2012).
In addition, it has to be mentioned that for clinical application, expansions beyond four passages lead to senescence with a decrease of proliferation accompanied with morphological and biological changes (Mocchi et al., 2020).

Questions about working with autologous or allogeneic cells always arise. Although using autologous cells for therapy might appear as the most appropriate material according to immunocompatibility, supply and ethics considerations, several drawbacks do exist. First, harvesting cells of a sick donor could

result in a reduction of clonogenic cells, proliferative rate, differentiation and angiogenic potential (Rozier et al., 2018). Moreover, approximately 3 weeks are necessary to obtain sufficient cell quantities from harvesting to implantation, therefore treating a patient in acute phase with autologous cells might not be possible (Zayed et al., 2018). On the other hand, exposing the patient to multiple allogeneic MSCs could be deleterious for diseases like OA because of the activation of adaptive immune response (Joswig et al., 2017). Indeed, allogeneic MSCs can express MHC II after engraftment and lose their immune privileged status (Huang et al., 2010).

Despite high immunomodulatory properties, side effects of cell therapy were also described. For instance, paracrine factors secreted by MSCs can interact with tumor cells, especially by increasing angiogenesis, which favors metastasis (Samsonraj et al., 2017). Aberrant differentiation is also a reported limitation for using MSCs (Driscoll & Patel, 2019). Therefore, a balance between benefit and risk of MSCs therapy must be measured.

2.2. Cell therapy in equine medicine

Cell therapy has several described applications especially for orthopedic conditions in horses (Voga, Adamic, Vengust, & Majdic, 2020). In addition to the well-known musculoskeletal applications, these cells are also used in equine ophthalmology, reproduction, metabolic disorders, respiratory systems, integumentary system, neurological disorders and endotoxemia (Cequier, Sanz, Rodellar, & Barrachina, 2021).

Several sources of MSCs have been described in the horse and are similar to humans: BM, adipose tissue, synovium, umbilical cord, tendon, muscle, gingiva or periodontal ligament (Ortved & Nixon, 2016). Harvesting BM-MSCs represents several complication risks: infection, hemorrhage and especially pneumothorax or pneumopericardium in the horse (Vidal et al., 2007). Adipose tissue offers an easier and safe access with rapid recovery after harvest. Umbilical cord blood is a readily available and highly proliferative source of horse MSCs but caution must be applied about the non-sterile conditions during parturition (Passeri et al., 2009).

Although several studies have been conducted with MSCs for horses, it is difficult to draw a coherent picture since they report highly variable results whatever the disease considered, depending on the source of MSCs, cell isolation procedure, culture condition or the administration routes (reviewed in (MacDonald & Barrett, 2020)).

Currently there is no central legislation for veterinary cell therapy in Europe (Voga et al., 2020). In Belgium, the use of stem cell therapy is regulated by the Agence Fédérale des Médicaments et des

Produits de Santé (AFMPS) and veterinary use is subject to the law related to drugs of 25 March 1964 (AFMPS, 2018): medicinal product need to obtain the AFMPS' approvement before marketing.

2.3. Osteoarthritis

Osteoarthritis (OA) is a degenerative disease that concerns over 10% of people over 60 years of age worldwide and etiology is multiple and includes genetics, metabolic factors, inflammation and trauma (Arrigoni et al., 2020). Knee and hip, two large weight-bearing joints, are the most commonly affected (He et al., 2020). The joint is a complex organ formed by several tissues (figure 1A). The proper function of this organ is conditioned by a finely tuned balance between anabolic and catabolic processes of these several joint constituents (Piluso et al., 2019).

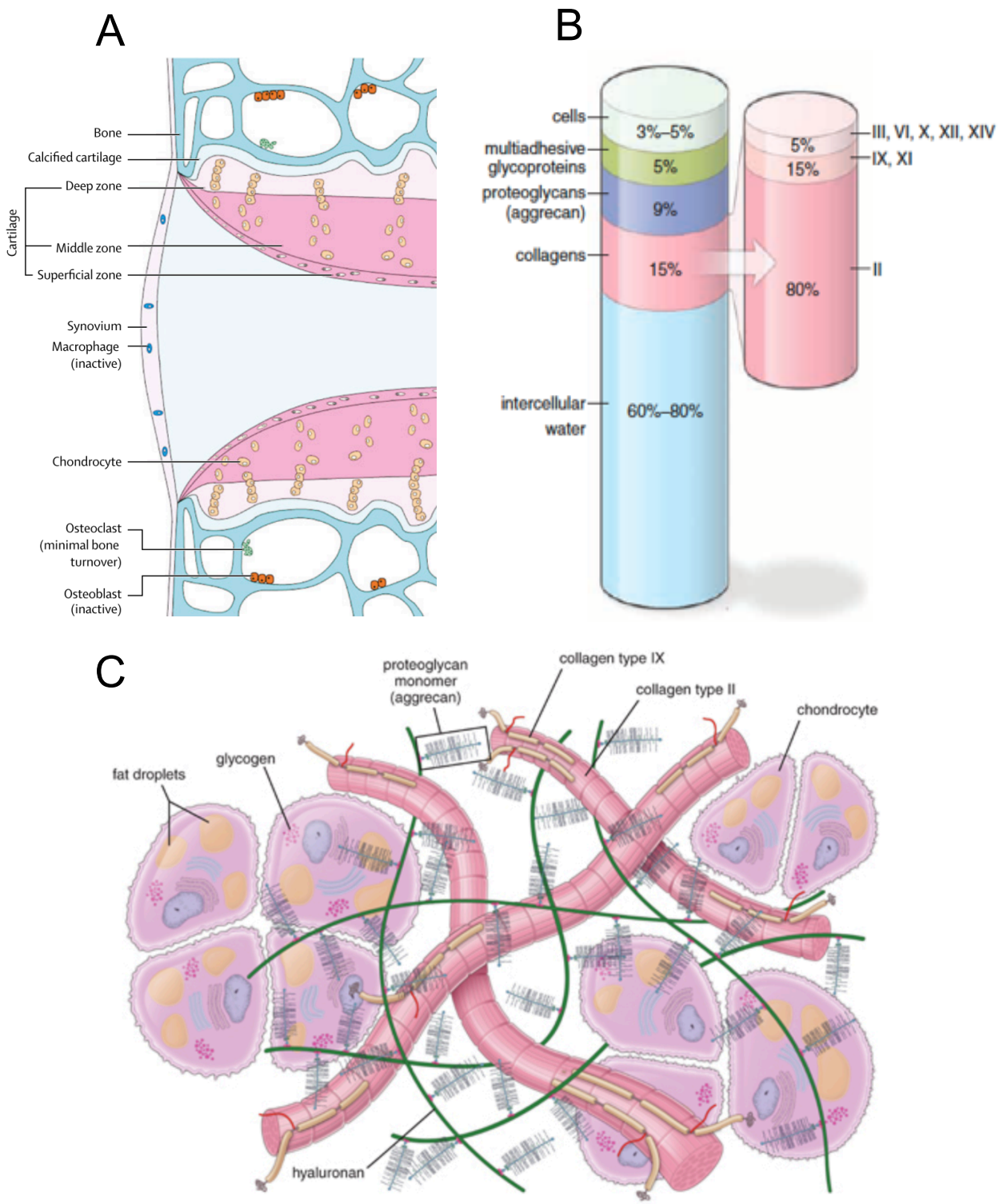

Figure 1 : A) Joint is composed of bone, cartilage, synovial membrane and liquid (Hunter & Bierma-Zeinstra, 2019). *B & C) Molecular composition (B) and structure (C) of hyaline cartilage* (Pawlina, 2019).

2.3.1.Joint description

- The **articular cartilage** is hyaline cartilage (figure 1B). It has different functions which depend on the developmental stage: cartilage is the template for long bone formation during growth and acts as shock-absorber for adult bone (Cohen, Foster, & Mow, 1998). The cartilage allows to

absorb and translate the force supported by the joint during movements thanks to its rich and very specific ECM (Thysen, Luyten, & Lories, 2015). Cells of this smooth and gliding surface are mostly chondrocytes but articular cartilage-derived progenitor cells have also been identified in cartilage (Alsalameh, Amin, Gemba, & Lotz, 2004). This population of progenitor cells represents up to 1% of cartilage cell content (Levato et al., 2017). Chondrocytes synthetize a very specific and abundant ECM (Charlier et al., 2019). The role of cartilage ECM is to confer tensile and compressive strengths properties (Poole et al., 2001). Chondrocytes are responsible for the synthesis and maintenance of the ECM (Zayed et al., 2018).

- The ECM production requires a high protein synthesis as evidenced by the large and highly developed Golgi apparatus and endoplasmic reticulum of chondrocytes (Armiento, Alini, & Stoddart, 2019). Chondrocytes produce mainly three types of collagen: II (90%), IX (1%) and XI (3%) (Eyre, 2002). In addition to these collagens, ECM is also composed of other proteins (figure 1C):
    - Proteoglycans: The shock-absorber function is conferred by the large volume of water bound to proteoglycans that are proteins modified with negatively charged glycosaminoglycans (GAG) (Armiento et al., 2019). Aggrecan is the major proteoglycan in cartilage (Q. Li et al., 2020). The core of this protein is linked to over 100 GAG molecules (Krishnan & Grodzinsky, 2018). In the cartilage ECM, proteoglycans such as aggrecan have the particularity to bind long hyaluronan molecules, by link proteins, to form large proteoglycan aggregates. These highly charged proteoglycan aggregates are bound to the collagen matrix fibrils that are associated with collagen network (Han et al., 2019). Versican and small leucine-rich proteoglycans (SLRP) such as decorin or lumican are other proteoglycans found in cartilage (Krishnan & Grodzinsky, 2018).
    - Fibers: In addition to the most prominent collagen type II, and to less abundant collagen types IX and XI, the ECM also contains collagen types III, V, VIII, X, XII, XIII, XIV and elastin (Gao et al., 2014).
    - Multi adhesive glycoproteins: Fibronectin and laminin are also identified in the ECM of cartilage (Gao et al., 2014). Other glycoproteins play different roles in the articular cartilage like clusterin, a glycoprotein implicated in immune response (Chen, Fu, Wu, & Pei, 2017).
- Several enzymes are implicated in the maintenance of ECM and can be classified according to their substrates: collagenases are matrix metalloproteinases (MMPs) 1, 8 and 13 (table 1); aggrecanases are a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTs) 4 and 5. Plasmin, a serine proteinase, is the active form of the pro-enzyme plasminogen and has several actions in cartilage: to cleave

fibronectin, proteoglycans or to activate proMMPs. Endogenous inhibitors of these proteinases are respectively tissue inhibitors of metalloproteinases (TIMPs) and Serine proteinase inhibitors (serpins) (Wilkinson, Arques, Huesa, & Rowan, 2019).

The narrow tissue surrounding the chondrocyte is called pericellular matrix (PCM) and forms the "chondron" together with the cells. PCM distinguishes from ECM by the presence of collagen type VI (Gao et al., 2014).

Cartilage is not vascularized and this particular environment has critical consequences on cell populations. First, chondrocytes have adapted their metabolism to anaerobic conditions (Buckwalter, 2002) and the articular cartilage is not able to self-renew (Jiang et al., 2016). Chondrocytes present a plasticity that allows them to modify their phenotype during disease and to become hypertrophic during OA for example (Charlier et al., 2019).

- The **subchondral bone** is protected by articular cartilage from forces on the joint surface (Ortved & Nixon, 2016).

- Joints are notably delimited by a **synovial membrane** (or synovium), composed by synoviocytes that produce the synovial fluid resulting from a mixture between plasma dialysate and hyaluronic acid (HA). The microstructure of this membrane is unique because fenestrations and pores allow the membrane to act as a sieve (Goodrich & Nixon, 2006). Cells form a single layer and a loose connective tissue with blood vessels and nerves to constitute the synovial membrane (Pujol et al., 2018). This membrane is supported by a outer fibrous layer which is the capsular ligament (Goodrich & Nixon, 2006).

- The **synovial fluid** has a function of cartilage nourishment by diffusion because cartilage is not vascularized. Another function of this fluid is to lubricate the articular surface (Zayed et al., 2018).

2.3.2. Osteoarthritis pathology

OA is a chronic condition that leads to irreversible tissue degradation due to inflammation (Zeira et al., 2018). Lesions are multiple and concern all constituents of the joint environment: cartilage degradation, synovial inflammation and bone lesions (osteophytes, subchondral sclerosis and BM lesions) (Lee et al., 2020) (figure 2). Synovial cells, immune cells, osteoblasts, chondrocytes or adipocytes are cells present in the joint and able to release inflammatory mediators contributing to the initiation and perpetuation of OA pathogenesis (Berenbaum, 2013). The early inflammatory response is influenced by the innate immune cells while cells of adaptive immunity lead to the chronic phase of the disease (Harrell, Markovic, et al., 2019). If components of cartilage's decomposition are exposed to B cells,

autoantibodies are produced and deposited in articular cartilage, making an immune complex that activates the complement system and induces tissue damage (Mödinger et al., 2019).

Pro-inflammatory cytokines activate inflammatory cells and promote the expression of hydrolytic enzymes such as MMPs (table 1), ADAMTs or cathepsin K (Bertuglia, Pagliara, Grego, Ricci, & Brkljaca-Bottegaro, 2016).

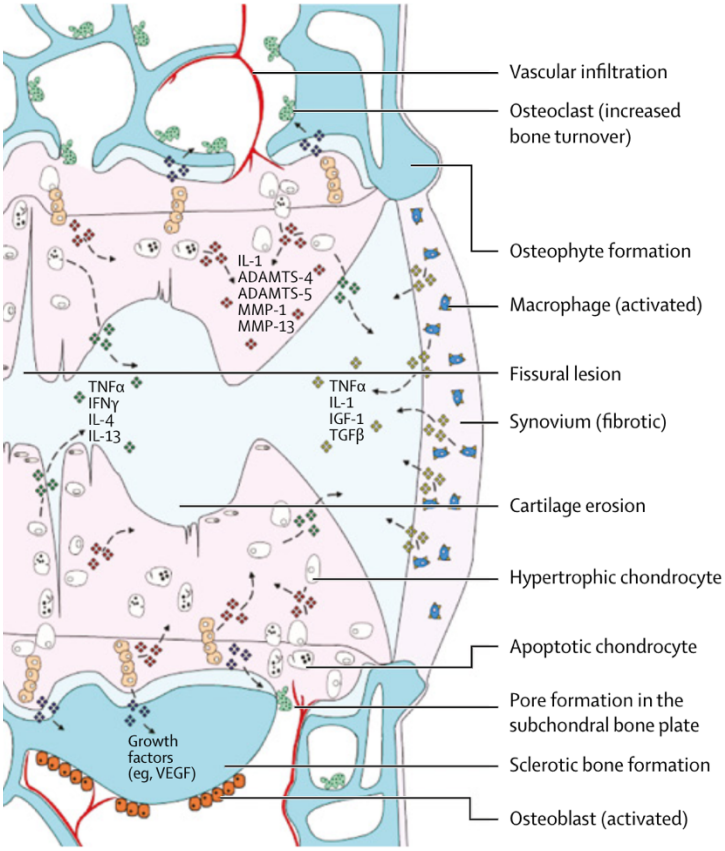

Figure 2: Consequences of OA on joint constituents and their molecular actors implicated in this pathology (Hunter & Bierma-Zeinstra, 2019).

Enzyme	Pseudonyms	Collagen Substrates	Additional Substrates
MMP-1	Collagenase-1	I, II, III, VII, VIII, X	Aggrecan, Gelatin
MMP-2	Gelatinase A	I, II, III, IV, V, VII, X, XI	Aggrecan, Elastin, Fibronectin, Gelatin, Laminin
MMP-3	Stromelysin-1	II, III, IV, IX, X, XI	Aggrecan, Elastin, Fibronectin, Gelatin, Laminin
MMP-4	Identified as MMP-3	-	-
MMP-5	Identified as MMP-2	-	-
MMP-6	Identified as MMP-3	-	-
MMP-7	Matrilysin	IV, X	Aggrecan, Elastin, Fibronectin, Gelatin, Laminin
MMP-8	Collagenase-2	I, II, III, V, VII, VIII, X	Aggrecan, Elastin, Fibronectin, Gelatin, Laminin
MMP-9	Gelatinase B	IV, V, VII, X, XIV	Aggrecan, Elastin, Fibronectin, Gelatin
MMP-10	Stromelysin-2	III, IV, V	Aggrecan, Elastin, Fibronectin, Gelatin, Laminin
MMP-11	Stromelysin-3	Aggrecan, Fibronectin, Laminin	-
MMP-12	Macrophage	IV	Elastin, Fibronectin, Gelatin, Laminin
MMP-13	Collagenase-3	I, II, III, IV	Aggrecan, Gelatin

MMP-14	MT1-MMP	I, II, III	Aggrecan, Elastin, Fibronectin, Gelatin, Laminin
MMP-15	MT2-MMP	Fibronectin, Gelatin, Laminin	-
MMP-16	MT3-MMP	-	-
MMP-17	MT4-MMP	Fibrin, Gelatin	-
MMP-18	Identified as MMP-19	-	-
MMP-19	RASI-1	IV	Aggrecan, Fibronectin, Gelatin, Laminin, COMP
MMP-20	Enamelysin	Aggrecan, Amelogenin, COMP	-
MMP-21	X-MMP	-	-
MMP-22	C-MMP	-	-
MMP-23	–	-	-
MMP-24	MT5-MMP	-	-
MMP-25	MT6-MMP	IV	Fibronectin, Gelatin, Laminin
MMP-26	Matrilysin-2	IV	Fibronectin, Gelatin
MMP-27	-	-	-
MMP-28	Epilysin	-	-

Table 1: Matrix metalloproteinase family contains several members with different substrates. MT: membrane type (Araki & Mimura, 2017).

2.3.3. Chondrocyte phenotype during OA pathogenesis

As mentioned before, chondrocytes participate in the OA pathogenesis, especially by a modified secretory phenotype. Matrix proteins, matrix proteases and matrix protease inhibitors, inhibin/activin or clusterin are differentially expressed molecules in the secretome of OA chondrocytes (Trachana, Mourmoura, Papathanasiou, & Tsezou, 2019). Matrix proteases, as MMP (summarized in the table 1), degrade the ECM by using different cartilaginous substrates. This modification of microenvironment leads chondrocyte to form clusters (Pearle, Warren, & Rodeo, 2005). This cell arrangement modifies the ECM secreted by chondrocytes, such as the decrease of proteoglycans production (Maldonado & Nam, 2013). This field is therefore a promising approach to identify new biomarkers linked to OA disease (Sanchez et al., 2017).

Another chondrocyte modification during the disease concerns mitochondria that present several dysfunctions participating in the pathogenesis. ROS increase is observed and explained by several mechanisms: decrease of the anti-oxidant molecules like superoxide dismutase 2, impairment of mitochondrial integrity and so in electron transport chain (Loeser, Collins, & Diekman, 2016). These dysfunctions participate in the increased chondrocyte catabolism.

2.3.4. Diagnosis

OA diagnosis is performed on clinical symptoms and radiographic findings (Liao et al., 2018), similarly in man and horse. The primary symptom of OA is pain (Pujol et al., 2018). Articular surfaces are not only non-vascularized structures, they are also an aneural tissues, which explains the low perception of pain when there is articular damage. On the contrary, synovial membrane, subchondral bone and periosteum are innervated structures that perceive and transfer pain signals (van Weeren & Back, 2016). Therefore, detection of early stages of OA is particularly challenging because early OA can be asymptomatic and due to the lack of sensitivity for minimal cartilage lesions on x-rays (Liao et al., 2018). X-rays-based diagnostic is limited to the detection of advanced lesions. Indeed, bone mineral

density must change at least by 30-40% to observe a modification on X-rays (C. Wayne McIlwraith et al., 2018).

The development of OA is slow, unpredictable and a shift exists between clinical signs and molecular events (McCoy, 2015). Proteomic research is currently seen as interesting field to identify candidate biomarkers, to highlight molecular mechanisms involved and to facilitate early OA diagnosis (Liao et al., 2018). The most promising indicator of MMPs activity is the biomarker from type II collagen degradation used both in human and animal models (Charni, Juillet, & Garnero, 2005).

2.3.5. Treatment

Principal therapies are symptomatic with administration of anti-inflammatory drugs. Surgery (joint replacement) is the only option available for joints with high grade of OA. The main challenge is to identify therapeutic strategies decreasing the progression of the disease and treating existing lesions (Thysen et al., 2015). Therefore, regenerative therapies based on stem cells are promising treatments for joints suffering from OA and several clinical trials showed that stem cell therapy can be a reliable treatment for this disease (Arrigoni et al., 2020).

2.3.6. Equine osteoarthritis

Major causes of retirement for sport horses are joint diseases and OA (Ortved & Nixon, 2016). Carpus and metacarpophalangeal joints are the most common affected joints of racehorse while the stifle is the most affected joint with OA for Western performance horses (C. W. McIlwraith, Frisbie, & Kawcak, 2012). OA concerns more than 50% of horses older than 15 (van Weeren & Back, 2016) and 60% of lameness is related to OA (Bertoni et al., 2020). Microtrauma, synovitis or a major joint trauma are probably the most frequent etiologies for equine OA. Lameness caused by OA presents an intermittent character with switches between symptomatic at asymptomatic phases (van Weeren & Back, 2016).

Curative treatment does not exist up to now in equine medicine, so management of the horse and traditional therapies are the main ways to act on the disease. Joint arthrodesis can be performed for horses suffering from end stage OA (Ortved & Nixon, 2016). Management principles include maintaining adequate body weight, assuring good housing conditions with soft surfaces, consider specific shoeing, balance joint stability with controlled exercise and treat every flare-up of OA (van Weeren & Back, 2016). Anti-inflammatory molecules used are mostly steroids for intra-articular way and non-steroid molecules for systemic delivery in horses. These treatments are cost effective and could quickly relief pain. Hyaluronic acid is used in equine medicine to increase the synovial liquid viscosity, to improve shock absorption and then to decrease inflammation (Azambuja da Silva, Pinto da Rosa, Mackmill, & Roll, 2021). Hydrogel is also used for synovial fluid substitute: this safe and lasting product reduces the lameness in horses suffering from OA (Tnibar et al., 2015).

Strategies leading to cartilage repair include several biological therapies. Autologous conditioned serum (also called Interleukin-1 Receptor Antagonist Protein (IRAP)) is obtained from PB and after different steps to concentrate endogenous molecules like IL-1 receptor antagonist (Linardi, Dodson, Moss, King, & Ortved, 2019). Platelet-rich plasma (PRP) is also described to contain regenerative factors from platelets like TGF-ß1 (Textor, Willits, & Tablin, 2013). Cell therapy in equine medicine is growing in interest with chondrocyte implantation, MSCs administration or bioengineered cartilage (Ortved & Nixon, 2016). Variable results are reported in treating naturally occurring OA in horse with MSCs because this disease is heterogeneous in terms of duration and severity (Mocchi et al., 2020). IRAP, PRP and stem cells are reported to be used at respectively 83,3%, 72,5% and 53,7% in a recent US study about joint therapy in equine practice (Zanotto & Frisbie, 2021).

2.4. Mesenchymal stem cells for osteoarthritis treatment

Treating OA with intra-articular injection of MSCs has been described to decrease joint inflammation and lead to cartilage regeneration in several animal species (such as mice, rats, pigs, horses or donkeys) (Harrell, Markovic, et al., 2019). Clinical studies have already been performed in human medicine with improvement of clinical outcomes (Jang, Lee, & Ju, 2021). Mechanisms of action of these cell therapies in the OA context are described below and are summarized in the figure 3.

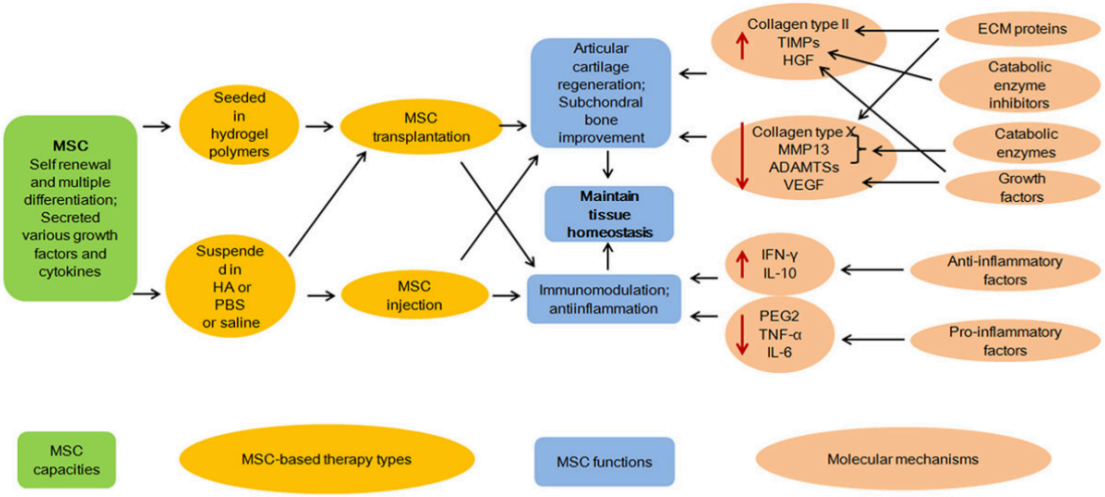

Figure 3: OA therapy based on MSCs helps to maintain articular tissue homeostasis by improving the cartilage regeneration and decreasing the inflammatory environment of the joint. Several MSCs secreted factors are involved to achieve these functions (R. Zhang, Ma, Han, Zhang, & Ma, 2019).

Therapeutic properties result from the capacity of MSCs to join the injury, to secrete trophic factors to allow regeneration and to modulate the inflammatory environment of OA. OA therapy should allow cartilage regeneration but also modulate immune cells activities according to their major impact in the pathology of this disease (X. Zhao et al., 2020).

2.4.1. Migration of mesenchymal stem cells

To reach the lesion sites, MSCs need to have efficient homing properties to migrate at the site of injury and exert their immunomodulatory properties (Nitzsche et al., 2017). First, migration is regulated by the presence of chemokines and their binding to MSCs chemokine receptors. Then, MSCs use their adhesion molecules like integrins to interact with cells. Finally, MMP in the extracellular matrix or secreted allow migration toward tissues (Naji et al., 2019).

2.4.2. Immunomodulation by mesenchymal stem cells

OA is not only a mechanical joint injury: inflammatory and immune systems are also implicated in this pathology. Macrophages, T and B cells are the main cells involved in OA immune response (X. Zhao et al., 2020). MSCs are able to interact with immune cells implicated in OA by cell-to-cell contacts (juxtacrine) or through the production of soluble factors (paracrine way) (Harrell, Markovic, et al., 2019) (figure 4).

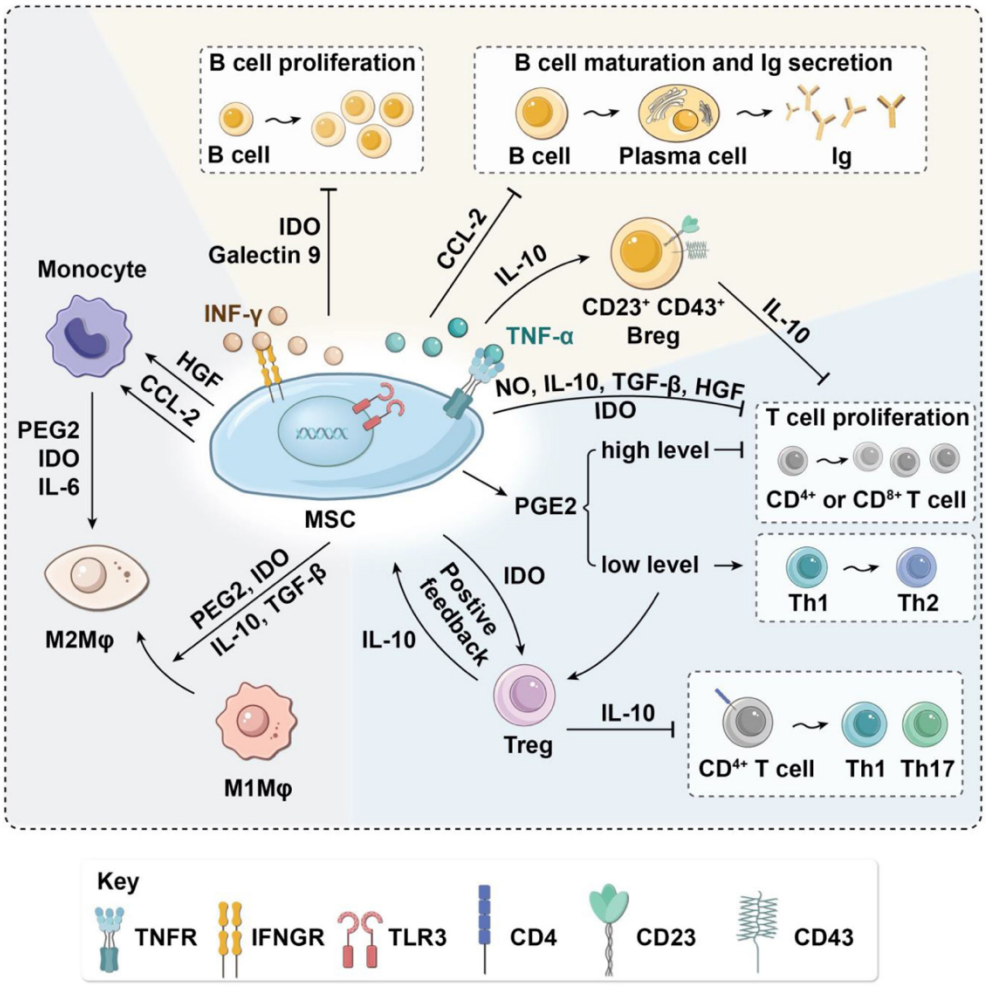

Figure 4: Pro-inflammatory cytokines like interferon gamma or TNF-α lead to the secretion of bioactive factors by MSCs and explain the modulation of MSCs toward immune cells by different mechanisms: regulation of macrophages polarization, inhibition of T cells proliferation, induction of immunosuppressive Treg and decreased B-cell activation . (X. Zhao et al., 2020)

These secreted factors constitute the secretome and are soluble proteins, nucleic acids, lipids or extracellular vesicles (EV) (Harrell, Fellabaum, et al., 2019).

- **Soluble factors**: The secretome of MSCs contains a plethora of soluble factors like ECM actors, cytokines, proteases or protease inhibitors (Mancuso, Raman, Glynn, Barry, & Murphy, 2019). Among others, anti-inflammatory factors (like IL-10) or anti-catabolic factors (like TIMPs) are secreted by MSCs in OA context (Richardson et al., 2016).

- **EV** are described to induce similar immunomodulatory and anti-inflammatory effects than parental cells. Therefore, their properties are already studied in several disease models. EV are surrounded by a lipid bilayer and are naturally secreted by cells to allow intercellular communication (Qiu et al., 2019). Size and origin of EV differ and are therefore classified as apoptotic bodies (>1000nm, formed during apoptosis), microvesicles (100-1000nm, of plasma membrane origin) or exosomes (30-200nm, formed during endosomal process) (Harrell, Fellabaum, et al., 2019). EV contain proteins, RNA and/or DNA and can induce signaling of recipient cells by endocytosis, fusion or binding to specific receptors. As non-cellular therapy, MSC-EVs present a low immunogenicity and good *in vivo* stability (Lai, Weng, Guo, Chen, & Du, 2019). However, EV-based therapy has several disadvantages reviewed in X. Zhao and al.: i) They are not able to reproduce the contact effect of MSCs; ii) EVs undergo a rapid clearance so local delivery is important ; iii) EV present variable effects due to several factors related to MSCs sources, isolation, culture, cryopreservation, … (X. Zhao et al., 2020)

  In OA context, chondrocytes can incorporate EVs from MSCs, leading to downregulate pro-inflammatory actors (like IL-6, IL-8, COX-2) and the release of proteases (Alcaraz, Compañ, & Guillén, 2019).

- **Fragmented, metabolically inactivated or apoptotic MSCs** also possess an immunomodulation potential (A. R. R. Weiss & Dahlke, 2019). By indirect effect, apoptotic cells decrease the reactivity of immune cells: apoptotic cells are phagocyted by macrophages which induces expansion of Treg cells and fail to induce CD4 T helper cells (Griffith et al., 2007). Moreover, dead or fragmented cells should have less tumor induction capacity than living MSCs (A. R. R. Weiss & Dahlke, 2019).

2.4.3. Chondroprotective effects of MSCs

Anti-inflammatory and chondroprotective effects of MSCs on chondrocytes are due to the decreased production of inflammatory mediators (TNF-α, IL-1β, IL-6, nitric oxide) and the enhanced production

of immunosuppressive molecules like IL-10 (Harting et al., 2018). The paracrine function of MSCs plays a major role in chondrocyte cellular processes including for examples:

- Expression of collagen type II and aggrecan could be increased while catabolic markers could be decreased by exposure to MSCs exosomes (Mianehsaz et al., 2019).
- Thrombospondin 2, a cartilage regulator, is secreted by MSCs and participates in the cartilage regeneration (Hankenson & Bornstein, 2002)
- Chondrocytes treated with MSCs exosomes upregulate the expression of anti-apoptosis gene like Survivin and Bcl-2 (S. Zhang et al., 2018)

2.5. Conclusions

Cell therapy based on mesenchymal stem cells is a promising approach to treat OA disease. This regenerative effect is mostly explained by the paracrine capacity of mesenchymal stem cells. However, cell therapy is influenced by several factors concerning the preparation and the way of administration of this product. It is therefore important to characterize the type of MSCs used in most relevant models of the disease studied.

Which model to assess the regenerative potential of cell therapy for osteoarthritis disease?

3. Which model to assess the regenerative potential of cell therapy for osteoarthritis disease?

3.1. Introduction

There are several experimental models described in literature to study OA. These models are both *in vivo* and *in vitro* with their respective advantages and disadvantage reviewed in the publication of He & al (He et al., 2020). Considering that the goal of this work is to characterize a new cell therapy product, *in vitro* model is prior to *in vivo* study.

After a short review of literature on *in vitro* models of OA, we will describe the different models we have tested with the aim to characterize the secretome of mdMSCs in conditions mimicking the pathology. In a first attempt we differentiated mdMSCs into chondrocyte-like. A second attempt consisted in 2D and 3D culture of chondrocytes isolated from horse cartilage. We finally selected the use of horse cartilage plugs exposed to IL-1β and TNFα to mimic inflammatory conditions.

3.2. *In vitro* models of osteoarthritis

There is currently no "gold standard" model of OA (Cope et al., 2019). Indeed, none of the described models is sufficiently robust and complete to make all the articular constituents and the actors of OA interact which each other (Haltmayer et al., 2019).

3.2.1. Chondrocytes

The predominant process in the pathology of OA is the progressive degradation of cartilage with a limited capacity of self-regeneration. As chondrocytes is the main cell type in cartilage, different *in vitro* model based on culture of chondrocytes were developed (He et al., 2020). The easy way to obtain these cells and the low cost of this model explain why it is still used a lot today. However this simple model does not represent the complexity of the joint involving interactions between multiple actors (Piluso et al., 2019).

Differentiation of MSCs in "chondrocyte-like cells" or digestion of cartilage explants are the 2 processes which allow to obtain chondrocytes:

- **Chondrogenic differentiation of MSCs** is induced by the supplementation of media with different growth factors implicated in this differentiation like dexamethasone and TGF-β. These factors will modify the morphology of MSCs into a more round shape and regulate the expression of genes implicated in chondrocyte phenotype (especially collagen type II/IX/XI and aggrecan) (Almalki & Agrawal, 2016).

Differentiation can be performed in monolayer or in 3D: by forming high-density pellet of cells or by using substrates favoring the homogenous distribution of cells. The high availability of these cells and the easy access, on the contrary to explants of natural tissue constitute significant advantages. The main challenge is to obtain the right phenotype of these differentiated cells: hypertrophic state often prevents the build-up of a functional articular hyaline cartilage (Somoza, Welter, Correa, & Caplan, 2014).

- **Primary articular chondrocytes**: Chondrocytes are obtained after enzymatic digestion of cartilage explants. This step leads to remove the rich ECM surrounding the cells. However, as ECM is implicated in several functions related to chondrocytes (like metabolism, cell signaling, differentiation,…) (Gao et al., 2014), its removal leads to alter the phenotype of chondrocytes. Moreover, opposite to MSCs, access to explants can be very limited depending on the organism considered. In addition, a high inter-donor biological variability is often observed (Brama et al., 2000).

  Chondrocytes can be cultured in different ways:

  - Monolayer cell culture: Dedifferentiation and loss of chondrocyte phenotype in culture are the main issues for this model. However, such modification of the phenotype is associated with OA (Thysen et al., 2015) so preliminary results could be obtained with this model which is considered as a first approach to study effects of an isolated event (Piluso et al., 2019).

  - 3D cell culture: this type of culture consists in cultivating the chondrocytes in spheres, which allows a better development of cartilage matrix than monolayer. 3D culture can be facilitated by using aggregate substrates like hydrogels, which influence cellular response to mechanical stimuli (Fahy, Alini, & Stoddart, 2018).

3.2.2. Explants

Ex vivo tissue culture allows to study and characterize chondrocytes in their physiological matrix limitation (Johnson et al., 2016). In contrary with 2D or 3D cell culture, cell microenvironment is conserved (Piluso et al., 2019), making this model closer to *in vivo* condition (Haltmayer et al., 2019).

These models can be more or less elaborate to study interaction of several joint constituents during co-culture as in particular between cartilage and synovial membrane explants (Haltmayer et al., 2019). Co-culture of different joint constituents leads to enhanced proteolytic activity demonstrating the synovium importance (Swärd et al., 2017).

However, despite the conservation of the ECM, culture can induce some phenotype modifications or cell death of at the cut edge of tissue. The availability of replicates is also a limitation (Johnson et al., 2016).

3.2.3. Microphysiological systems

Models described above are static models which cannot mimic mechanical stimuli as tension, compression and shear stress (Piluso et al., 2019). Microphysiological systems are *in vitro* models associating 3D culture of cells and dynamic tissue crosstalk (figure 5). They allow to work with living cells in a specific microenvironment with structure and responses close to the studied tissue (He et al., 2020). These models are relevant to predict the efficacy of therapeutic drugs as OA in joint is influenced by dynamic processes (compression, vascularization,…) (Occhetta et al., 2019).

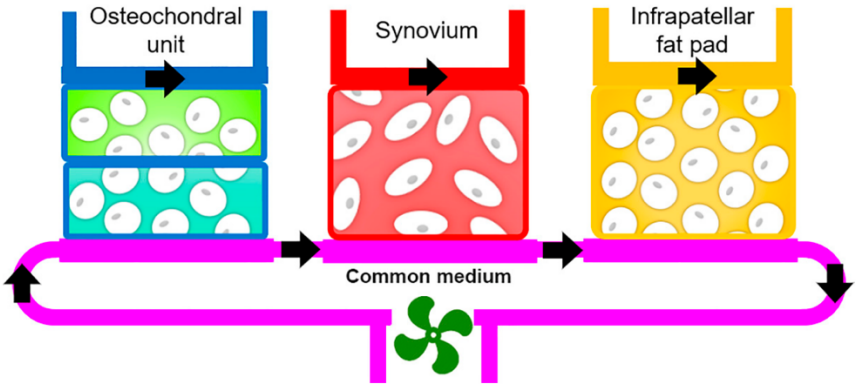

Figure 5: Example of a microphysiological system allowing to mimic the interaction between the different joint components (He et al., 2020)

3.2.4. Inflammatory part of osteoarthritis model

Inflammatory processes are induced mechanically (compressions) or chemically (cytokines supplementation) in these *in vitro* models (Haltmayer et al., 2019). IL-1β and TNFα are described as the best cocktail to induce an inflammatory reaction similar to OA (Cope et al., 2019). A lot of data is already published about culture of cartilage explant with these cytokines (table 2). Concentration of IL-1β and TNFα is around 0,1ng/mL in the synovial fluid of patients with OA (Matta et al., 2020).

Material	Pro-inflammatory model	Effects	References
Bovine cartilage explant	TNFα (100 ng/mL) or IL-1β (10 ng/mL)	GAG release, nitrite production, MMP3 and 9 secretion ↑ in the expression of MMP1, MMP3, MMP9, complement factors B/C3/C1r, IL-6, CCL2, CCL20, CCL5, M-CSF, CXCL6, CTGF, osteopontin, semaphoring 3C, neuropilin 2, pleiotrophin, sFRP1 and 4, IGFBP 3 and 5	(A. L. Stevens, Wheeler, Tannenbaum, & Grodzinsky, 2008) (Anna L. Stevens, Wishnok, White, Grodzinsky, & Tannenbaum, 2009)
Coculture of cartilage explant, bone and synovial membrane (human)	IL-1β (10 ng/mL)	↑ TNF-α, IL-6Rα, IL-8, IL-15, CCL-1, CCL-2, CXCL-10, MMP-3 and MMP-13	(Kardos et al., 2019)
Rat cartilage explant	IL-1β (20 ng/mL)	↑ in TNF-α, PGE2, MMP-13, GAG, NO_2^-, ↓ in TIMP-1	(Pathak et al., 2015)
Bovine cartilage explant	TNFα (100 ng/mL) or IL-1β (100 ng/mL)	GAG release and degradation of aggrecan	(Tortorella, Malfait, Deccico, & Arner, 2001)
Canine cartilage explant	IL-1β (20 µg/mL)	GAG release	(Siengdee, Pradit, Chomdej, & Nganvongpanit, 2019)
Bovine cartilage explant	TNFα (100 ng/mL) or IL-1β (10 ng/mL)	GAG release	(Stradner et al., 2011)
Human cartilage explant	IL-1β (10 ng/mL)	GAG release, secretion of pro MMP1, total MMP3, pro MMP13	(Weinmann et al., 2018)
Human cartilage explant	IL-1β (1 or 10 ng/mL)	IL-6 and IL-8 production	(Van Offel et al., 2005)
Horse cartilage explant	TNFα (100 ng/mL) or IL-1β (10 ng/mL)	GAG (aggrecan catabolites) and hydroxyproline releasing	(Little, Flannery, Hughes, Goodship, & Caterson, 2005)
Bovine cartilage explant	TNFα (10 ng/mL)	Collagen release	(Hui et al., 2010)
Human cartilage explant	IL-1β (5 ng/mL)	TNFα, aggrecanase, ADAMTs-4 and 5, MMP1 and 13 production	(Huh et al., 2009)
Bovine cartilage explant	IL-1β (10 ng/mL)	GAG release, NO production	(Hartog et al., 2008)
Human cartilage explant	TNFα (5 ng/mL) or IL-1β (5 ng/mL)	COMP fragment production	(Luan et al., 2008)
Horse cartilage explant	TNFα (10 ng/mL) & IL-1β (10 ng/mL)	↑GAG release, ↑ COMP, ↓clusterin release	(Matta et al., 2020)
Horse cartilage explant	TNFα (10 ng/mL) & IL-1β (10 ng/mL)	↑: MMP1, MMP3, COMP, coagulation factor XIII A chain, Enolase 1, fibromodulin, lamin A/C, vimentin ↓: fibronectin, collagen type VI α, collagen type X α1 chain, matrix gla protein	(Anderson, Phelan, Foddy, Clegg, & Peffers, 2020)
Horse cartilage explant	IL-1β (10 ng/mL)	↑: MMP1, MMP3, thrombospondin 1	(Clutterbuck et al., 2011)

Table 2: Review of cartilage explants cultured in-vitro with IL-1β and TNF-α at different concentrations and molecules identified in their supernatants in these culture conditions.

IL-1β and TNFα are synthesized by chondrocytes, osteoblasts, cells of synovial membrane and mononuclear cells (Wojdasiewicz, Poniatowski, & Szukiewicz, 2014) (figure 6). These cytokines act on chondrocytes by inducing the release of enzymes like MMPs and aggrecanases (McAllister, Chemaly, Eakin, Gibson, & McGilligan, 2018) or by stimulating the oxide nitric production which contributes to cartilage degradation and chondrocyte cell death (Charlier et al., 2016). Synthesis of pro-inflammatory molecules like IL-6, IL-8, prostaglandin E2 (PGE2) by chondrocytes, synovial cells and T lymphocytes is also stimulated by IL-1β and TNFα (Belluzzi et al., 2019; Calich, Domiciano, & Fuller, 2010).

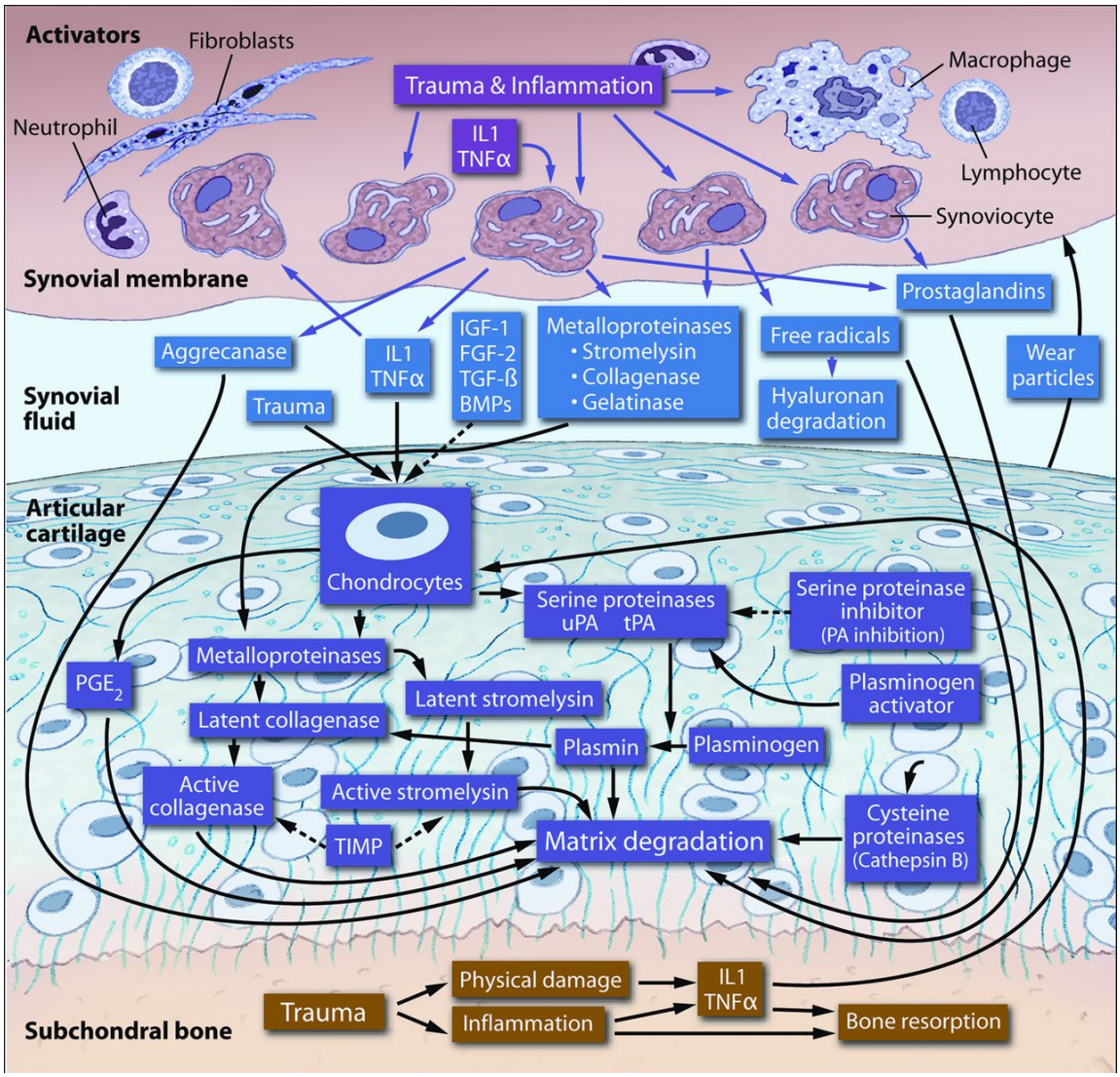

Figure 6: IL-1β and TNF-α are produced by chondrocytes, synoviocytes or immune cells and lead to activate catabolic metabolism in the different constituent of the joint: synovial membrane, articular cartilage and subchondral bone. Dotted lines are inhibitor factors of the degradation occurring during OA disease (C. W. McIlwraith et al., 2012).

3.3. Horse as a model specie for osteoarthritis

The horse is an athletic species with a relatively long lifespan similar to human (Bundgaard et al., 2020). Horses are considered being the more anatomically similar animal model to humans to study articular

therapy before clinical trials (Bapat et al., 2018). Horse joints share a lot of common features with humans as articular cartilage thickness (Peffers, Cillero-Pastor, Eijkel, Clegg, & Heeren, 2014). Average cartilage thickness is between 1.5-2mm for horses and 2.2-2.5mm for humans (McCoy, 2015). Cellular structure and biochemical composition of horse cartilage are two other features very comparable to humans (Malda et al., 2012). Horse stifle is described to be similar to human knee (Cope et al., 2019).

As mentioned previously, OA prevalence is naturally high for this species (C. Wayne McIlwraith et al., 2018) because racehorses are submitted to repetitive impacts and therefore to joint injuries during their athletic career (Bertuglia et al., 2016). Moreover, a lot of data elements are already published for this species: clinical tests, imaging and rehabilitation techniques for horses suffering from osteoarthritis are described (Strauss, Goodrich, Chen, Hidaka, & Nixon, 2005). Arthroscopic surgery can be performed given that the large size of the joint allows visual observation of the inner part of the joint (Bapat et al., 2018). These elements make OA a well-known and characterized disease in horse.

Working with animal models constitutes an advantage about access to tissue because human tissues are often only available after joint replacement at the end stage of the disease when little can be concluded (McCoy, 2015). Finally, using spontaneous animal pathologies like OA in horses is a strategy to limit the use of experimental animal models (Mocchi et al., 2020).

3.4. Material & Methods

3.4.1. Differentiation of mdMSCs to chondrocytes

The equine skeletal mdMSCs were provided by RevaTis (Aye, Belgium). They were cultured in Dulbecco's modified Eagle's medium (DMEM) F-12 culture medium (Gibco) supplemented with 20% heat-inactivated fetal bovine serum (HI-FBS) (Gibco), 100 IU/mL of penicillin-streptomycin (Gibco) and 0.5% of amphotericin B (Gibco) at 37°C and 5% CO_2 according to the recommendations of RevaTis (Ceusters et al., 2017).

MSCs differentiated into chondrocytes can be obtained in several ways. We studied 2 of them: differentiation of mdMSCs in monolayer or in spheres.

- For monolayer differentiation, mdMSCs were cultured until 70% of confluency after which culture media was changed to StemProTM Chondrogenesis Differentiation (Gibco) media for 21 days.
- For the differentiation in spheres, mdMSCs were detached with trypsin, washed and then 250 000 cells were placed into a falcon 15mL with 1 mL of the same chondrogenesis differentiation media for 21 days (Ceusters et al., 2017).

At the end of the differentiation period, cells were washed with Phosphate-Buffered Saline (PBS) and processed for alcian blue staining or for mass spectrometry (MS) analysis.

For alcian blue staining, cells were fixed with paraformaldehyde (PFA) 4% for 30 minutes and then were colored with a solution of 1% alcian blue in 0.1N HCl buffer like described in the manual furnished by Gibco with StemPro Chondrogenesis Differentiation Kit.

3.4.2. Mass spectrometry analysis

After extraction of proteins in buffer containing urea 7M, thiourea 2M, CHAPS 1%, ASB14 1%, Tris 30mM, sodium dodecyl sulfate (SDS) 1% at pH 8.5, protein concentration was determined with Pierce assay (Thermo Fisher, 22660) and 35 µg were processed. Digestion was performed with filtered-aided sample preparation (FASP) that consists in diluting samples in UA buffer (Tris-HCl 0.1 M, Urea 8 M, pH 8.5), reducing proteins with dithiothreitol (DTT) 8 mM, alkylating them with iodoacetamide (IAA) 50 mM and digest protein with trypsin (Promega) (1 µg of trypsin for 50 µg of proteins) on Microcon Ultracel PL-30 (Millipore). After final concentration of the samples with speed vac, samples were diluted in acetonitrile 2 % and trifluoroacetic acid (TFA) 0.1% (final concentration 500ng/µL) before being frozen at -80 °C until MS analysis.

Peptides were analysed by using nano-LC-ESI-MS/MS maXis Impact UHR-TOF (Bruker, Bremen, Germany) coupled with a nanoLC UltiMate 3000 (Thermo). The digests were separated by reverse-phase liquid chromatography using a 75 µm X 250 mm reverse phase Thermo column (Acclaim PepMap 100 C18) in an Ultimate 3000 liquid chromatography system. Mobile phase A was 95 % water/5 % acetonitrile, 0.1 % formic acid. Mobile phase B was 20 % water/80 % acetonitrile, 0.1 % formic acid. The digest (18 µL) was injected, and the organic content of the mobile phase was increased linearly from 5 % B to 40 % in 90 min and from 40 % B to 100 % B in 10 min. The column effluent was connected to a Captive Spray (Bruker). In survey scan, MS spectra were acquired for 0.5 s in the m/z range between 50 and 2200. The 15 most intense peptides ions 2^+ or 3^+ were sequenced. The collision-induced dissociation (CID) energy was automatically set according to mass to charge (m/z) ratio and charge state of the precursor ion. MaXis and Thermo systems were piloted by Compass HyStar 3.2 (Bruker).

Peak lists were created using DataAnalysis 4.2 (Bruker) and saved as MGF file for use with Mascot 2.5.1 as search engine (Matrix Science). Enzyme specificity was set to trypsin, and the maximum number of missed cleavages per peptide was set at two. Carbamidomethylation, oxidation of methionine and hydroxylation of lysine or proline were allowed as variable modifications. Mass tolerance for monoisotopic peptide window was 7 ppm and MS/MS tolerance window was set to 0.05 Da. The peak lists were searched against the Equus database (from UNIREF) with an automatic decoy database search.

Scaffold 4.8 (Proteome Software) was used to validate MSMS-based peptide and protein identifications. Peptide identifications were accepted if they could be established above 95% probability by the peptide prophet algorithm (Keller, Nesvizhskii, Kolker, & Aebersold, 2002) with Scaffold delta-mass correction. Protein identifications were accepted if they could be established above 5 % probability to achieve a false discovery rate (FDR) less than 1 % and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, Keller, Kolker, & Aebersold, 2003).

3.4.3. Primary chondrocytes

Plugs of cartilage were collected (detailed in the next point called "cartilage sample collection"), washed and cut. Digestion was performed with an enzymatic cocktail containing Dispase II (Roche, 04942078001) and Collagenase D (activity 0.015UI; Sigma, 11088858001) in Dulbecco's Modified Eagle Medium (DMEM) F12 media with 10% of fetal bovine serum (FBS) at 37°C and 5% of CO_2 for 24 hours. TrypLE Express (Gibco) was added to the digestion medium in a ratio of 6:1 for 15 minutes. Cells were filtered on 70µM filters and this filtered suspension was then centrifuged (1000 RPM for 5 minutes at room temperature (RT)). Cells were counted and seeded into 24 wells plate with 10,000 cells per well. Cells were cultured at 37°C with 5% of O_2 with medium renewal (DMEM-F12 with 20% of FBS) every 2 to 3 days.

3.4.4. Cartilage sample collection

Plugs of cartilage were harvested on horses euthanized for other reasons than osteoarthritis disease and after obtaining owners' consent. Briefly, cartilage was obtained from stifle joint, with a scalpel blade (size 20), on femoral trochlea through a sterile access to the joint caudal to the lateral patellar ligament. Plugs of cartilage were weighed and placed in the same culture medium as mdMSCs during approximately 4 days before their use for experimental procedures.

Thin slices of cartilage were cut and then incubated with 2 mL of PBS – Ethidium Bromide (final concentration of 10 µg/mL) (Sigma-Aldrich, E8751-1G) and Acridine Orange (final concentration of 3 µg/mL) solution in 2-well Chambered Coverglass Nunc Lab-Tek (Thermofisher) for 30 min at 37 °C and protected from light. Then, red and green fluorescences were observed with confocal microscopy (SP5, Leica) by using Z stacking.

3.4.5. Nuclear translocation of NF-kB (P65 immunofluorescence)

Cells were washed with PBS before fixation in 4% PFA for 10 minutes. Cells were permeabilized with 0.1% Triton X100 for 5 minutes and then incubated with 2% of bovine serum albumin (BSA) for 30 minutes. Incubation with the primary antibody (CSD14E12, 1:400) was performed overnight (O/N) at

4°C. Cells were rinsed 30 minutes with BSA 2% and then incubated with the secondary antibody (Alexa Fluor 488-conjugated anti-rabbit IgG antibody; Molecular Probes, A11034) and TOPRO-3 for 40 minutes at RT and protected from the light. Coverslips were mounted with Mowiol (Sigma) and observed with confocal microscopy (SP5, Leica).

3.4.6. Nuclear translocation of NF-kB (Western blot analysis of nuclear fractions)

Nuclear fractions were obtained as described by Renard et al., (Renard et al., 2001): cells were washed 2 times with cold PBS, scrapped and centrifuged for 10 minutes at 1000 rpm and 4°C. Lysis buffer (20 mM HEPES pH 7.5, 0.35 M NaCl, 20% glycerol, 1% NP-40, 1 mM $MgCl_2 \cdot 6H_2O$, 0.5 mM EDTA, 0.1 mM EGTA) was applied on the pellet for 10 minutes on ice before additional centrifugation (20 minutes at 14,000rpm and 4°C). Supernatant was harvested and protein concentration was determined with Pierce assay (Thermofisher). 5µg of proteins were incubated at 95°C for 5 minutes and then resolved by SDS polyacrylamide gel electrophoresis on a 12% gel, transferred with Transblot Turbo (Biorad) to a polyvinylidene fluoride (PVDF) membrane (ThermoFisher). The membrane was blocked 1 hour at RT with Odyssey Blocking buffer (Li-Cor Biosciences) followed by overnight incubation with the primary antibodies (table 3) diluted in blocking buffer with 0,1% of Tween 20 (Bio-Rad Laboratories). After 3 washes with PBS-tween 2%, the incubation with the secondary antibody was performed for 1 hour followed by 3 washes with PBS-tween 2%. The membrane was scanned with odyssey Infrared Imager (Li-Cor Biosciences) and fluorescence was quantified with the Odyssey V3.0 software (Li-Cor Biosciences). TBP detection was used as a loading control and for normalization.

Protein	Antibody	Primary or secondary	Dilution
P65	CSD14E12	Primary	1/1000
TBP	SC204	Primary	1/1000
Rabbit	R700, Licor, 926-68071	Secondary	1/10 000

Table 3: Antibodies used for western blot analysis of NF-kB nuclear translocation.

3.4.7. RT-qPCR

Total RNA was extracted with ReliaPrep RNA Miniprep Systems (Promega) as recommended by the manufacturer. 2µg of total RNA was reverse transcribed using GoScript Reverse Transcription mix Oligo(dT) (Promega) following supplier's instructions. GoTaq qPCR Master Mix (Promega) was used to perform amplification on a Viia7 equipment (Applied Biosystems). Primers listed in table 4 were used at a concentration of 300nM. All the results were normalized to the mRNA abundance of enolase using the $2^{\Delta\Delta Ct}$ method and expressed relative to the untreated cells condition. Data was analyzed using the Kruskal-Wallis test followed by the Dunn's multiple comparison.

Gene	Forward	Reverse	Efficiency	Source
Enolase	GTGCAGCCAACTTCAGTGAA	CCAGCTTTCCCAATGGCATT	97%	Personal
Coll αI (type II)	CTGAAACTCTGCCACCCTGA	TGCTCCACCAGTTCTTCTTGG	95%	Personal
Coll αI (type I)	GATGTGCCACTCTGACTGGA	GTACCAGACGTGCCTCTTGT	98%	Personal

Table 4: Primers used for qPCR

3.4.8. Phenotypic analysis of cartilage plugs: response to inflammatory cytokines

- **Nitrite dosage**: After the centrifugation of the supernatant (5 min, 1000 rpm, at RT), 50 µL of each sample were used to measure the NO_2^- with the Griess system reagent (Promega). Nitrite dosage was performed in duplicate according to the manufacturer's instructions. Absorbance was measured at 535 nm with spectrophotometer (xMark, Bio-Rad). Results were normalized on the mass of plugs.

- **Glycosaminoglycan release dosage**: Blyscan sulfated glycosaminoglycan assay (Biocolor) was performed on 10 µL of supernatant according to the manufacturer's recommendations. Results were normalized on the mass of plugs.

- **IL-6 ELISA**: The levels of secreted IL-6 in cell culture supernatant were assessed using an Equine IL-6 DuoSet ELISA (R&D Systems, United Kingdom) according to manufacturer's instructions. Results were normalized on the mass of plugs.

- **Statistical analysis**: Statistical analyses were performed with Prism (GraphPad Software) using Wilcoxon tests.

3.5. Results: selection of the cellular component of the OA *in vitro* model

To study the potential therapeutic impact of MSC on joint component, we had to first define an appropriate *in vitro* model composed of both cellular and inflammatory components. As chondrocytes are probably the most impaired cellular actor during OA, we considered 3 different ways to produce chondrocytes: i) chondrocytes derived from the differentiation of mdMSCs, ii) primary chondrocytes isolated from cartilage and iii) cartilage explants.

The main criteria considered for selecting the best chondrocytes production method were the quality of phenotype (close to *in vivo* chondrocytes), the reproducibility, the robustness and the material supply.

3.5.1. Differentiation of mdMSCs in chondrocyte-like cells

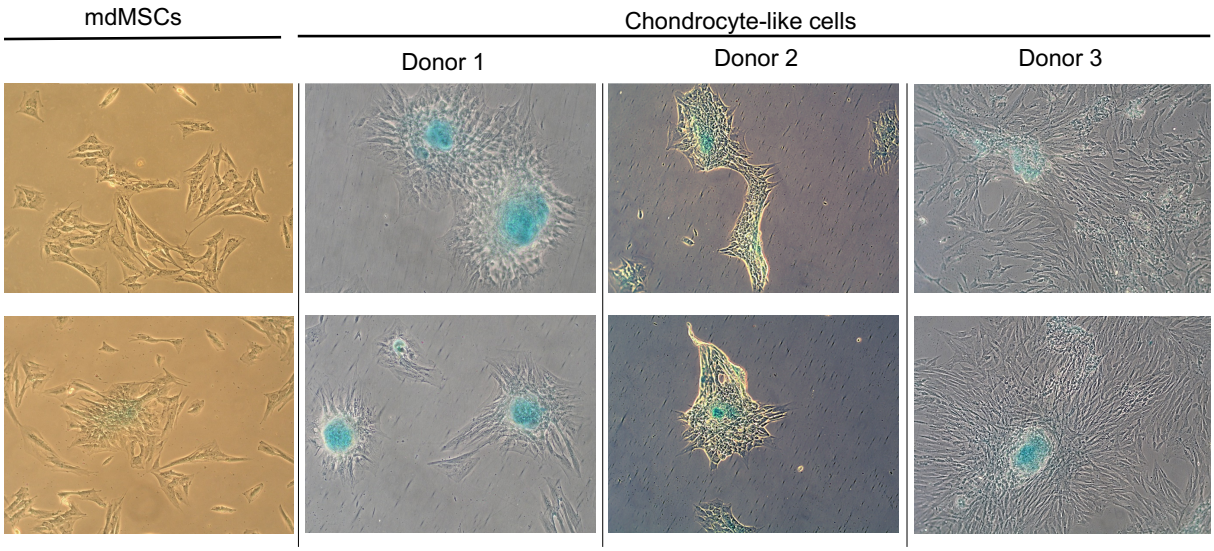

Figure 7: Differentiation of adherent mdMSCs in chondrocytes after 21 days of chondrogenesis differentiation media – Margaux Colin, ULB. After this period, cells were fixed and colored with alcian Blue before that mdMSCs non differentiated and differentiated were observed with microscope. Magnification: x300.

Differentiation of adherent mdMSCs with chondrogenesis media for 21 days is an easy method to obtain "chondrocyte-like" cells. The differentiation efficiency was assessed by staining the cells after fixation with alcian blue, a glycosaminoglycan dye (figure 7). Microscopically, we observed a high variability between biological replicates: shape of cells, confluency and intensity of the alcian blue dye.

Due to the difference between biological replicates, the few round cells and the low alcian blue staining, we decided to differentiate mdMSCs in 3D chondrocyte-like cells, hereafter called pellet (figure 8) (Ceusters et al., 2017).

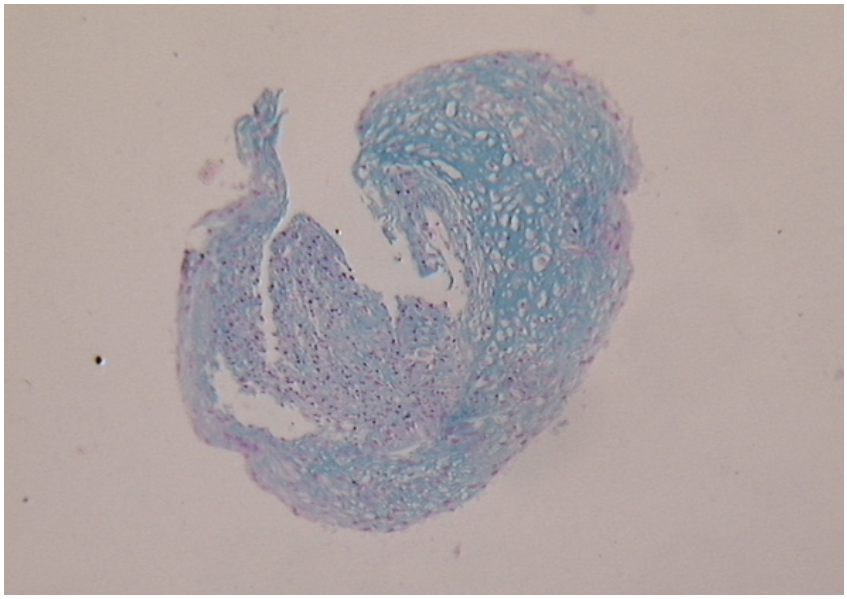

Figure 8: mdMSCs in pellet following 21 days of chondrogenic differentiation process (Ceusters et al., 2017).

Moreover, alcian blue dye is a subjective method to evaluate the differentiation of mdMSCs into chondrocyte-like cells. We therefore used proteomic analysis to evaluate the phenotype induced by these 2 ways of differentiation. In addition to these 2 samples, we analyzed an explant of cartilage harvested on euthanized horse and a sample of non-differentiated mdMSCs. The goal of this analysis was not to be quantitative but to determine if differentiation of mdMSCs with chondrogenesis media, in monolayer or in pellet, allows to identify collagens specific to articular cartilage.

Proteomic analysis of differentiated mdMSCs was performed. The most abundant collagen in cartilage, collagen type II, is only detected for mdMSCs differentiated in 3D, like described by Prosser and al., (Prosser, Scotchford, Roberts, Grant, & Sottile, 2019) and reported by Peffers and al., (Peffers, Collins, Loughlin, Proctor, & Clegg, 2016). The other 2 most abundant collagens are type IX, not detected in differentiated mdMSCs, and XI which is spotted for monolayer of differentiated mdMSCs as shown in the table 5. Collagen type VI α2 and α3 were also more sequenced in differentiated than non-differentiated MSCs as in the study of Rocha (Rocha et al., 2012). Despite the coherence of results with other teams, none of them did use a sample of cartilage as positive control to assess the evolution of differentiation.

Type of collagen	Monolayer of differentiated mdMSCs	Pellet of differentiated mdMSCs	Cartilage	mdMSCs
II α1		+	+++++	
III α1	+	++	+++	+
VI α1	+	+	+++++	
VI α2	+	+	++++++	
VI α3	+	+	+++++	
VI α6				
IX α1				
IX α2				
IX α3				
X α1				
XI α1	+		+++++	
XI α2				
XII α1	+	+++++		

Table 5 : Identification by mass spectrometry analysis of collagens in mdMSCs left undifferentiated or differentiated in 2D or 3D, and in plug of cartilage. The most abundant collagens, in bold, are type II, IX and XI. Detected collagens are highlighted in blue in the table while not detected collagens are in grey. "+" are indicators of the relative abundance between all samples. n=1

Therefore, taking into account the variability between biological replicates, the duration of differentiation and the low acquisition of chondrocyte phenotype, we suggested that this method is not robust enough to obtain chondrocyte-like cells allowing to further study the interaction between mdMSCs and cartilage.

3.5.2. Culture of chondrocytes

As differentiation of mdMSCs showed poor acquisition of chondrocyte markers, the culture of primary chondrocytes from plugs of cartilage was set up. After enzymatic digestion of cartilage explants, cells were filtered, counted and plated. Chondrocytes were cultured until 90% of confluency and then passaged (splitting 1:4; at 12, 19, 26, 39 and 50 days post collection of explants). Cells were maintained in culture until passage 5. At each passage, a sample of cells was harvested to extract RNA and to measure the expression of collagen type I and II by RT-qPCR. This method and the microscopically observation of cells allow to follow the phenotype of chondrocytes during culture.

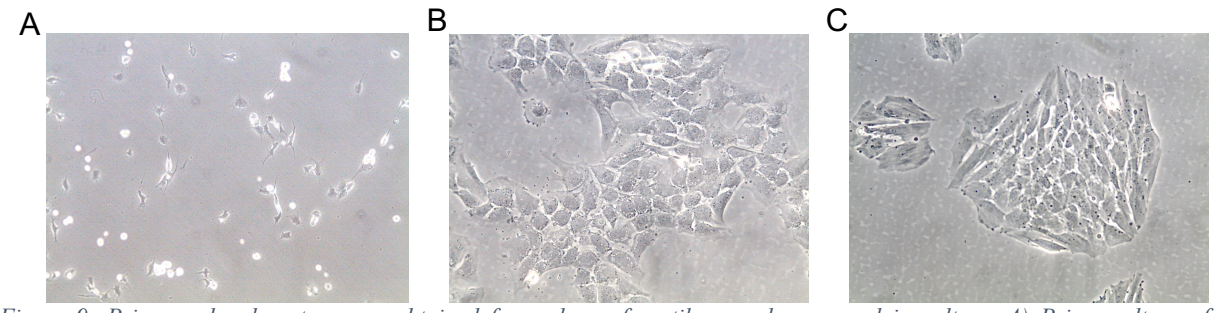

Figure 9: Primary chondrocytes were obtained from plugs of cartilage and conserved in culture. A) Primo culture of chondrocytes 2 days after seeding, magnification x100. B) Chondrocytes after 17 days of culture and 2 passages, magnification x200. C) Culture of cells 50 days after digestion and 5 passages, magnification x200.

As shown in figure 9, phase contrast microscopy observation of cultured cells phenotype showed that cell morphology is modified along cell culture and passages: the shape of cells became less round and proliferation of cells decreases after 4 passages. To monitor the chondrocyte phenotype of cells during cell culture, gene expression analysis of type I and type II collagens was performed. As reported by (Charlier et al., 2019), the shift of the ratio between collagen of type II and I is an indicator of dedifferentiation state of chondrocytes. Indeed, type II collagen is the main collagen of cartilage while type I collagen is a marker of dedifferentiated chondrocytes.

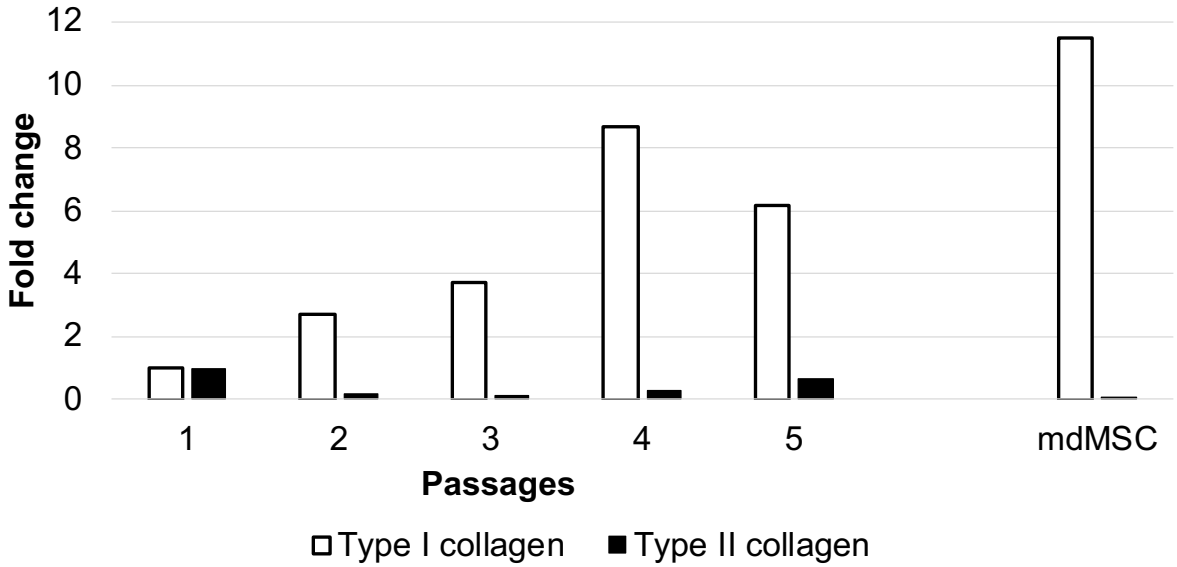

Figure 10: Expression of Type I and Type II collagen mRNA during culture of primary chondrocytes from passage 1 to 5, obtained by RT-qPCR. Gene expression of alpha 1 collagen (I) and alpha 1 collagen (II) was normalized to enolase expression and expressed as fold changes to chondrocytes at passage 1. n=1

Along passages, chondrocytes display an increased expression of collagen type I and a decrease of the collagen type II expression (figure 10). Evolution of the collagen type II/type I ratio suggests that these cells lose their phenotype during the cell culture (Benya, Padilla, & Nimni, 1978). Furthermore, the many steps to obtain these cells from plug of cartilages represent a high risk of contamination. These 2 reasons led us to consider the feasibility to work directly with plugs of cartilage.

3.5.3. Plugs of cartilage

Plugs of cartilage represent an opportunity to work with chondrocytes embedded in their matrix. The preservation of the environment of chondrocytes should limit their dedifferentiation (Johnson et al., 2016) and to study the behavior of mdMSCs in a closer joint model than chondrocytes alone.

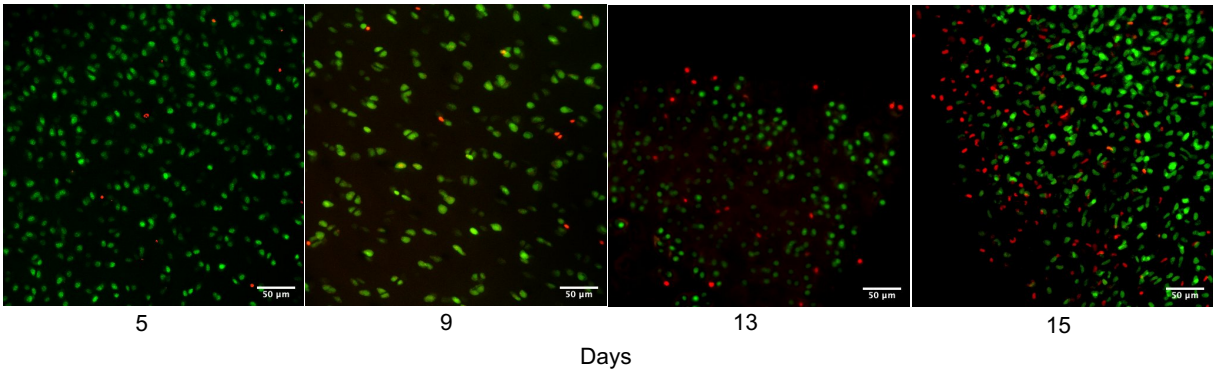

Figure 11: Assessment of chondrocyte viability during ex-vivo culture of cartilage plugs during 15 days after sample collection. Ethidium bromide stains dead chondrocytes (red) while acridine orange labeled living cells in green. Confocal microscopy analysis shows that most of the chondrocytes are still alive. Magnification 400x.

Explants of cartilage were already studied by several teams showing especially that after approximately 2 weeks of culture, explants were morphologically intact (Strehl, Tallheden, Sjögren-Jansson, Minuth,

& Lindahl, 2005), collagen content remained stable (Moo, Osman, & Pingguan-Murphy, 2011) but chondrocytes death concerns the edge of the explant (Gilbert et al., 2009). Therefore, ethidium bromide acridine orange incorporation was used to assess the viability of chondrocytes present in cartilage plugs cultured *in vitro* (figure 11). Chondrocytes are kept alive in plugs conserved in culture until at least 15 days after sampling with a mortality of approximately 15%. Despite many attempts to extract the RNA from explants, we failed to perform it. The goal of this step would have been to evaluate the phenotype of chondrocytes cultured in explant.

In conclusion of this articular set up part, we chose to work with plugs of cartilage, considering the easy way to obtain plugs, the low number of steps needed to work with them in culture, the viability of chondrocytes and the preservation of cartilaginous matrix with this method.

3.6. Results: selection of the inflammatory component of the OA *in vitro* model

After the identification of the best method to study the interaction between mdMSCs and chondrocytes with plugs of cartilage, we developed the inflammatory model by using TNF-α and IL-1β, described as the best method to mimic OA-induced cartilage damage (Cope et al., 2019).

We first identified which recombinant cytokines were able to induce a pro-inflammatory phenotype on equine cells. As these two cytokines are reported to trigger the expression of pro-inflammatory genes through the activation of the transcription factor NF-kB, the subcellular localization of this protein was assessed first by immunofluorescence analysis (figure 12A). Indeed, while NF-kB has a cytosolic localization at the basal state, it is translocated into the nucleus in response to pro-inflammatory cytokines (Mitchell, Vargas, & Hoffmann, 2016). Micrographs of the figure 12A show that both human cytokines induce the nuclear translocation of the Nuclear factor Kappa B (NFκB) P65 subunit on the contrary to non-treated mdMSCs where P65 is sequestered in the cytoplasm.

As the equine cells are responsive to human cytokines, a cocktail of both cytokines at low concentration was administered to cells as these 2 cytokines are identified in joint suffering from OA (Haseeb & Haqqi, 2013). We choose a low concentration compared to other research groups (table 2) because we wanted to mimic the chronic process of OA, for which TNF-α and IL-1β concentrations are around 0.1ng/mL in the synovial fluid (Matta et al., 2020).

Western blot analysis of enriched nuclear fractions of mdMSCs (figure 12B), indicates the nuclear localization of p65 in response to TNF-α and IL-1β treatment. Results of qPCR showed that TNF-α and IL-1β, respectively used at 1ng/mL and 0.1ng/mL, induced the expression of pro-inflammatory genes (IL-8 and IL-6), both in mdMSCs and primary chondrocytes (figure 12C) compared to non-treated cells.

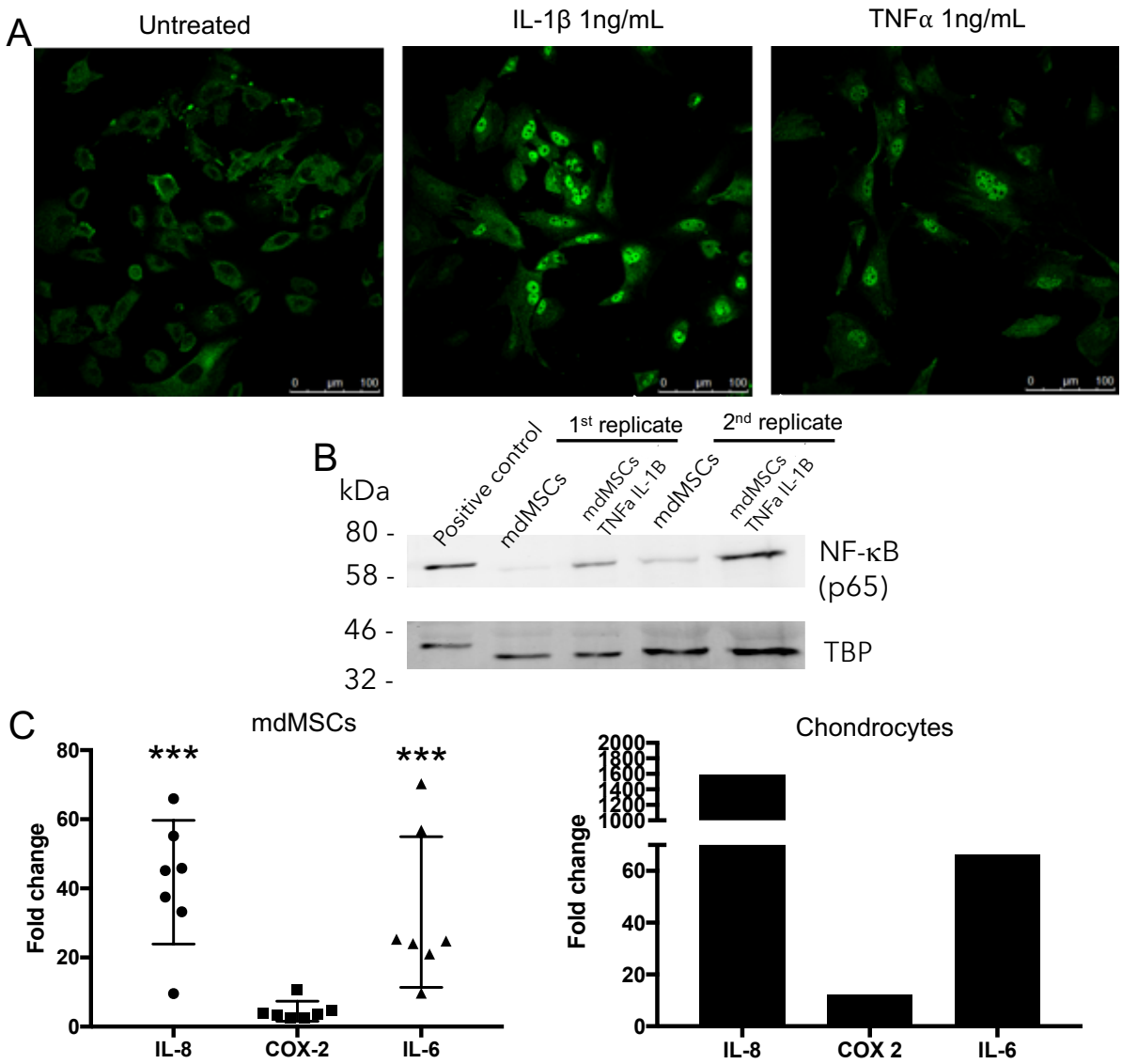

Figure 12: human TNF-α and IL-1β trigger an inflammatory response in equine mdMSCs and chondrocytes.

A: immunofluorescence analysis of the NF-kB P65 subunit in non-stimulated or stimulated mdMSCs with IL-1β or with TNF-α for 1 hour and at 1ng/mL. Magnification x200.

B: Western Blot analysis of NF-kB p65 subunit in enriched nuclear fractions of mdMSCs non treated or treated with TNF-α (1ng/mL) and IL-1β (0.1ng/mL) for 1 hours. Detection of TBP is used as a loading control. Positive control is a sample of murine RAW cells treated with 20ng/mL of LPS for 1 hour and already characterized to assess the nuclear translocation of P65.

C: Gene expression analysis of IL-8, COX-2 and IL-6 in mdMSCs and primary chondrocytes at passage 4 and treated for 24 hours with TNF-α (1ng/mL) and IL-1β (0.1ng/mL) and measured by RT-qPCR. The results are expressed in fold change related to untreated cells. P values were calculated following Kruskal-Wallis tests. n=7 for mdMSCs and n=1 for primary chondrocytes.

The response of plugs of cartilage to this pro-inflammatory cocktail was also studied. As explained previously, TNF-α and IL-1β exposure leads to release GAG, nitrite (NO_2^-) and IL-6 in the supernatant of cartilage plugs as a consequence of the activation of metalloproteinase, the stimulation of oxide nitric

production and the synthesis of pro-inflammatory cytokines. A significant increase of these inflammatory markers (figure 13) was highlighted after 3 days of TNF-α and IL-1β exposure.

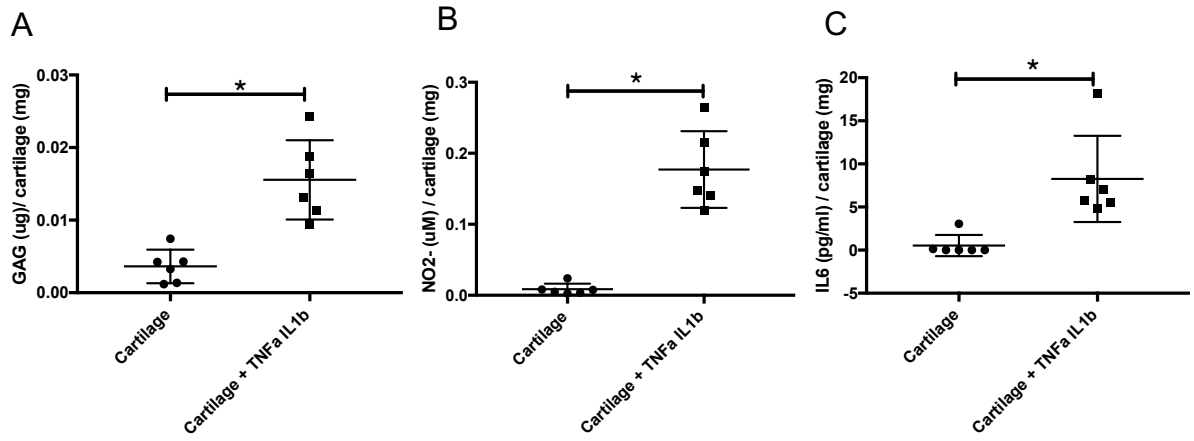

Figure 13: Pro-inflammatory phenotype of plugs of cartilage treated with TNF-α and IL-1β for 3 days showed the releasing of GAG in supernatant (A), the secretion of NO_2^- (B) and IL-6 (C). P values were calculated following Wilcoxon tests. n=6

3.7. Discussion

OA disease affects 250 million people around the world and surgery, the only available curative treatment, is invasive and must be reserved for appropriate candidates (Hunter & Bierma-Zeinstra, 2019). Therefore, stem cell therapy is seen as a promising approach to offer an alternative treatment to joint replacement. Although several treatments based on stem cell therapy are available for human, there is currently no consensus about the best use of these products. Many variables influence the therapeutic potential and more precisely the source of cells, the way to manipulate these cells before therapy and the injection conditions (injection route, number of cells, solvent, …) (Mocchi et al., 2020). Despite many positive results reported about MSCs therapy in equine OA, there is currently no guidelines for this specie, partly due to a large heterogeneity in cell therapy conditions: cellular origin, isolation, process, route of administration, dosing, … (Zayed et al., 2018). It is therefore essential that every product developed for stem cell therapy must be characterized in an appropriate *in vitro* model first.

The goal of this work being to study the potential of mdMSCs for OA treatment in horses, we focused on the development of a suitable and relevant *in vitro* OA model in perspective to use this model to study mdMSCs joint therapy.

As cartilage degradation is considered as a major mechanism in OA disease, we focused on this cell component for our model. Differentiation of mdMSCs into chondrocytes was not convincing regarding the variability between donors and the low degree of differentiation. Similar findings were shown by the group of Lin who reported limited differentiation efficiency and donor variability (Lin et al., 2019). In a second approach, primary chondrocytes were cultured to work with cells with a phenotype closer to the physiological situation. However, culture conditions seem to lead to dedifferentiation (Abbott &

Holtzer, 1966). Furthermore, the risk of contamination was particularly high to consider this method as enough robust to study the impact of MSCs on chondrocytes. Finally, we have worked with a straightforward approach to collect and study plugs of cartilage from euthanized horses' stifle. This technique allows to work with chondrocytes embedded in their matrix, a favoring element to maintain the chondrocyte phenotype (Hall, 2019). Despite many efforts to obtain RNA from plugs to extend the characterization of cell phenotype during plug culture, we were not able to obtain enough material to assess the phenotype by RT-qPCR. The low number of cells relative to cartilage weight, the difficulty of grinding this material and to work at low temperature could explain the failure of this step. However, other readouts suggest that cartilage plugs are appropriate and robust biological material to further investigate putative effects of MSCs. Indeed, chondrocytes are kept alive for at least 2 weeks and the plugs are able to produce an expected response to inflammatory environment, as described by other teams with the production of nitrite and the release of GAG and IL-6 (Cook, Cook, & Stoker, 2018; Madzuki, Lau, Che Ahmad Tantowi, Mohd Ishak, & Mohamed, 2018; Matta et al., 2020). Furthermore, considering the high variability between donors (mainly age and disease before euthanasia) (donors details are in section 7.2), results showed consistency between replicates.

Once the relevant chondrocyte model was selected, we developed the inflammatory medium with a mix of TNF-α and IL-1β, given that these cytokines are described to be the best method to induce lesions similar than OA *in vitro* (Cope et al., 2019). We used these cytokines at relatively low concentrations compared to other studies (table 2) because osteoarthritis is a chronic disease with low-grade inflammation (Van Den Bosch, 2021). This cytokine cocktail was sufficient to induce inflammatory response on both mdMSCs, chondrocytes and plugs of cartilage as shown by the high expression of IL-6 and IL-8 in mdMSCs and chondrocytes and by the releasing of GAG, the production of NO_2^- and IL-6 in supernatant of explants.

This model could be improved by involving other joint actors such as synovial membrane that host macrophages considered as key regulatory cells in inflammation for OA (Van Den Bosch, 2021). Therefore, it is a compound of the joint to consider which could improve our *in vitro* model.

3.8. Conclusions

In this second part, we have developed an *in vitro* model of osteoarthritis by setting up articular and inflammatory components representative of OA. We have shown that the *ex vivo* culture of cartilage plugs is a reliable method to assure the viability of chondrocytes in its matrix during culture conditions. Then, we have shown that exposure to a cytokine cocktail composed of TNF-α and IL-1β induces a pro-inflammatory phenotype in mdMSCs, primary chondrocytes and plugs of cartilage. The selected osteoarthritis model for further investigation therefore consists in using *ex vivo* plugs of cartilage in medium containing TNF-α (1ng/mL) and IL-1β (0.1ng/mL).

mdMSCs as a drug delivery device

5. mdMSCs as a drug delivery device

5.1. Introduction

Curcumin, also known as diferuloymethane, is a polyphenolic nutraceutical isolated from the roots of Curcuma longa (Abdollahi, Momtazi, Johnston, & Sahebkar, 2018). Curcumin is the major curcuminoid present in turmeric (Gupta et al., 2013) at a concentration of 2-5% in dried turmeric powder (Nakagawa et al., 2014).

Traditional Indian and Chinese medicines use this spice to treat inflammatory diseases for thousands of years (Priyadarsini, 2009). Curcumin is a hot topic with over 15 000 manuscripts related to the biological interactions and effects of curcumin (Nelson et al., 2017). The first medical effect of curcumin was published in 1937 and concerned the treatment of cholecystitis in human (Oppenheimer, 1937). In veterinary medicine, first publications supporting pharmacological activities of curcumin were published in the 90s (Lans & Brown, 1998).

Various biological effects of curcumin are conferred by its ability to modulate multiple cell signalling molecules (Gupta et al., 2013). Curcumin act as an anti-inflammatory molecule by inhibiting the activation of NFκB and the nitric oxide synthase (iNOS) enzyme (Alves et al., 2019).
The inhibition of NFκB leads to a down-regulated expression of pro-inflammatory cytokines such as Interleukin (IL)-1, IL-6, IL-8 and TNF-α (Y. Wang, Tang, Duan, & Yang, 2018). *In vitro* cytotoxicity of curcumin was reported for doses superior than 25 μM of curcumin for 48 hours (J. Zhao, Sun, Ye, & Tian, 2011).

Antioxidant properties of curcumin result from the structure of the molecule that confers an excellent electron transfer capability (Barzegar & Moosavi-Movahedi, 2011). The chemical structure of curcumin is two methoxy phenols attached symmetrically which confers photophysical and photochemical properties (figure 23) (Priyadarsini, 2009).

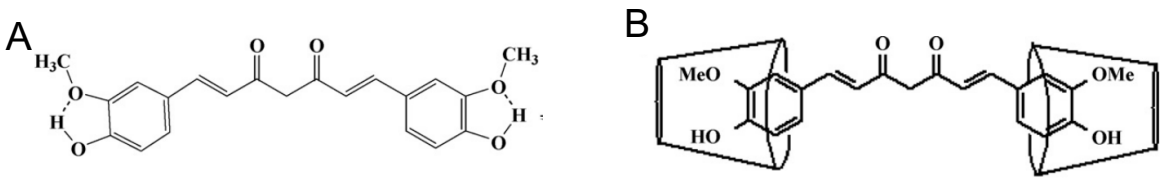

Figure 23: Chemical curcumin structure (A). Curcumin surrounded by 2 cyclodextrin complexes (B) (Priyadarsini, 2009).

Curcumin is widely studied as nutraceutical for osteoarthritis disease. Positive effects on pain and joint stiffness are described after an oral treatment with curcumin (Zeng, Yang, Hao, Yu, & Chen, 2021). Intra-articular injection of curcumin is also effective to decrease the inflammatory response induced by

OA: curcumin downregulates the expression of TLR4 and the NFκB pathway (D. Yan, He, Guo, Li, & Wang, 2019).

Despite its many biological effects, the major disadvantages of curcumin are its very poor water solubility and systemic bioavailability (Priyadarsini, 2009). The chemical instability (Metzler, Pfeiffer, Schulz, & Dempe, 2013) and the lipophilic nature of this molecule are responsible of these drawbacks. Curcumin is soluble in organic solvents like ethanol, methanol, dimethyl sulfoxide-acetone and others. The stability of curcumin is another concern : *in vitro* studies showed degradation up to 90% in physiological solution after one hour (Alves et al., 2019).
The European Food Safety Authority Panel set the acceptable daily dose of curcumin: 3mg/kg bodyweight (Alves et al., 2019). Human patients with pancreatic cancer were treated with 8g/day of curcumin and this polyphenol was safe and well tolerated (Dhillon et al., 2008). A negligible absorption and a low bioavailability explain the rare side effects of curcumin even when administered at high oral dose (Nelson et al., 2017).

Various strategies have been employed to increase this polyphenol bioavailability and to take advantages of its pharmaceutical effects: using adjuvants, nanoparticles, liposomes, micelles, phospholipid complexes or by changing the route of administration (Kunnumakkara et al., 2017). Using curcumin as an efficient drug is a challenge to develop a good drug candidate with properties like chemical stability, high water solubility, potent and selective target activity, high bioavailability, broad tissue distribution, stable metabolism and low toxicity (Nelson et al., 2017).

To associate cell therapy with curcumin, treatment of stem cells has been studied and showed different effects on mesenchymal properties. First, curcumin interacts with cell membrane proteins of MSCs and can activate or suppress different signaling pathways, such as Wnt or NFκB signaling pathways influencing the differentiation of MSCs into the three mesodermal lineages (Gorabi et al., 2019). Then, curcumin pre-treatment is an approach to increase the viability of MSCs during transplantation by the improvement of mitochondrial function (X. Wang et al., 2019).

The present study is focused on a water-soluble derivative of curcumin called NDS27 which is a hydroxypropyl-beta-cyclodextrin (HPβCD) complex of curcumin lysinate. Indeed, NDS27 is about 33,000 times more soluble in water than synthetic curcumin (WO/2009/144220, 2009). In addition, cyclodextrin complexes could improve the penetration of curcumin into cells compared to the native form (Derochette et al., 2014). The goal of this association is to increase the immuno-modulatory capacity of mdMSCs with a derivative curcumin form by acting on antioxidant and paracrine properties in inflammatory conditions. Previous study shows that NDS27 is able to inhibit superoxide anion production by neutrophils (Franck et al., 2019). The association between MSCs harvested in muscle

with minimally invasive process and a curcumin derivative, that does not require any organic solvent to be dissolved, may lead to a new therapeutic tool suitable for clinical application. The characterization of this cellular product was described in the publication (Colin et al., 2021) listed below. This cryopreserved product was developed to be used intra-articularly in horses joint suffering from OA and injected directly post thawing.

5.2. mdMSCs as drug carrier for NDS27

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SHORT COMMUNICATION

WILEY

Priming of mesenchymal stem cells with a hydrosoluble form of curcumin allows keeping their mesenchymal properties for cell-based therapy development

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Abstract

Mesenchymal stem cells are increasingly studied for their use as drug-carrier in addition to their intrinsic potential for regenerative medicine. They could be used to transport molecules with a poor bioavailability such as curcumin in order to improve their clinical usage. This natural polyphenol, well-known for its antioxidant and anti-inflammatory properties, has a poor solubility that limits its clinical potential. For this purpose, the use of NDS27, a curcumin salt complexed with hydroxypropyl-beta-cyclodextrin (HPβCD), displaying an increased solubility in aqueous solution, is preferred. This study aims to evaluate the uptake of NDS27 into skeletal muscle-derived mesenchymal stem cells (mdMSCs) and the effects of such uptake onto their mesenchymal properties. It appeared that the uptake of NDS27 into mdMSCs is concentration-dependent and not time-dependent. The use of a concentration of 7 μmol/L which does not affect the viability and proliferation also allows preservation of their adhesion, invasion and T cell immunomodulatory abilities.

KEYWORDS

curcumin, cyclodextrin, equine MSCs, mdMSC, mesenchymal stem cells, mitochondria, NDS27

Margaux Colin and Lola Dechêne should be considered joint first authors

Didier Serteyn and Véronique Mathieu should be considered joint senior authors

1 | INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent stem cells implicated in homeostasis and tissue repair.[1] Curcumin, a polyphenol extracted from the roots of *Curcuma Longa* L., is used to treat notably inflammatory diseases for thousands of years.[2,3] The major disadvantages of curcumin are its very poor water solubility and its low systemic bioavailability.[3] The present study is therefore focused on a hydroxypropyl-beta-cyclodextrin (HPβCD) complex of curcumin lysinate called NDS27, which is about 33 000 times more soluble in water than synthetic curcumin.[4] The association of MSCs with a complex of curcumin that does not require any solvent to be dissolved, may lead to a new therapeutic tool suitable for clinical application. Thus, this study aims to evaluate (a) the cellular toxicity of the NDS27 on MSCs, (b) the uptake and subcellular localization of NDS27 and (c) the effects of such loading (ie uptake) on mitochondrial function and mesenchymal properties of skeletal muscle-derived MSCs (mdMSCs).

2 | MATERIALS AND METHODS

Detailed materials and methods are provided in the Appendix S1 section.

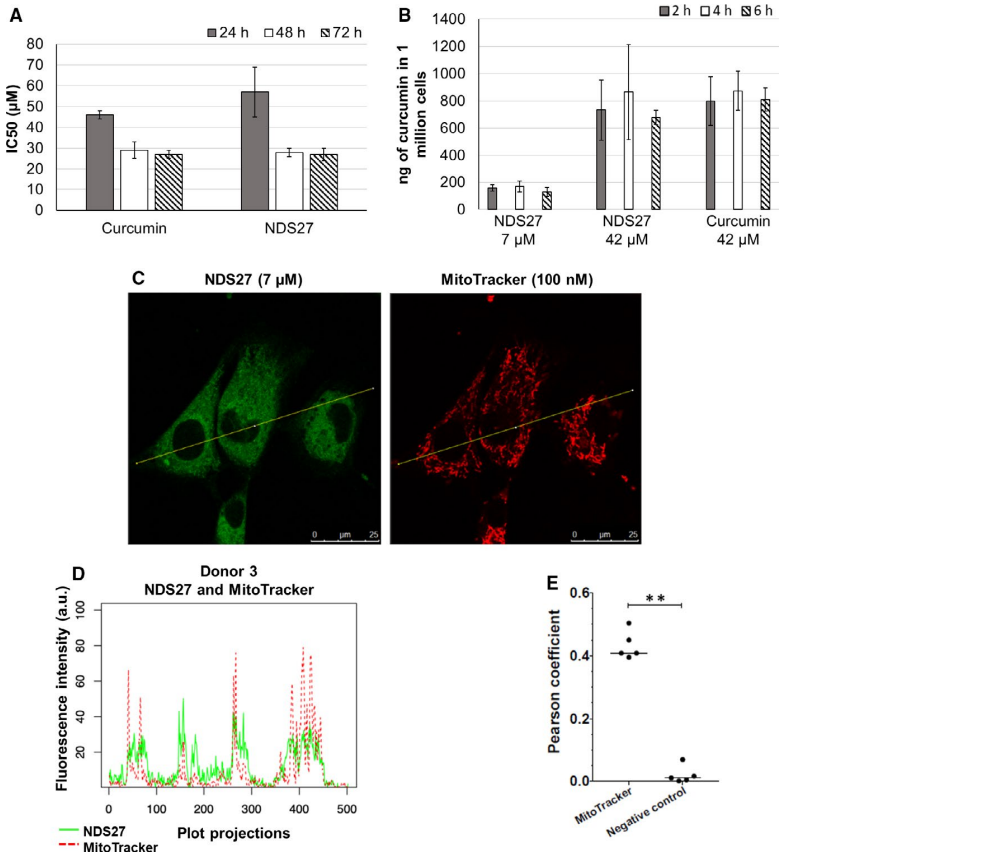

FIGURE 1 Internalization of NDS27 and synthetic curcumin by equine mdMSCs. (A) Average IC_{50} of synthetic curcumin and NDS27 on equine mdMSC after 24, 48 or 72 h of treatment relative to untreated control. Data are expressed as mean ± SD of five independent biological replicates. (B) Quantity of curcumin (ng) internalized in one million equine mdMSCs after 2, 4 or 6 h of treatment with NDS27 (7 or 42 µmol/L) or synthetic curcumin (42 µmol/L). Data are expressed as mean of three independent biological replicates ± SD. (C) Micrographies of mdMSCs from donor 3 after their loading with NDS27 at 7 µmol/L (green fluorescence; Ex: 488 nm; Em: between 502-576 nm) and staining with MitoTracker probe (red fluorescence; Ex: 561 nm; Em: between 572-636 nm) (magnification ×400). The thin yellow lines present on the representative micrography is the transect used for the co-location evaluation. (D) Intensities of both green (NDS27) and red (MitoTracker) fluorescence for each point along the transect traced on the micrography shown in (C), indicating that NDS27 is partially co-located with MitoTracker and thus, mitochondria. The presented result was obtained on donor 3 and is representative of experiments conducted on two other donors. (E) Data from 5 different donors (at least 6 images/donor) were quantified to assess the correlation of co-location between natural fluorescence of NDS27 and MitoTracker probe using Pearson coefficient method. The correlation between NDS27 and MitoTracker is significantly distinct from the correlation between NDS27 and control condition (consisting in micrography of MitoTracker probe rotated of 90°) suggesting that the mitochondrial co-location of NDS27 is not random. $P < .01$ (**)

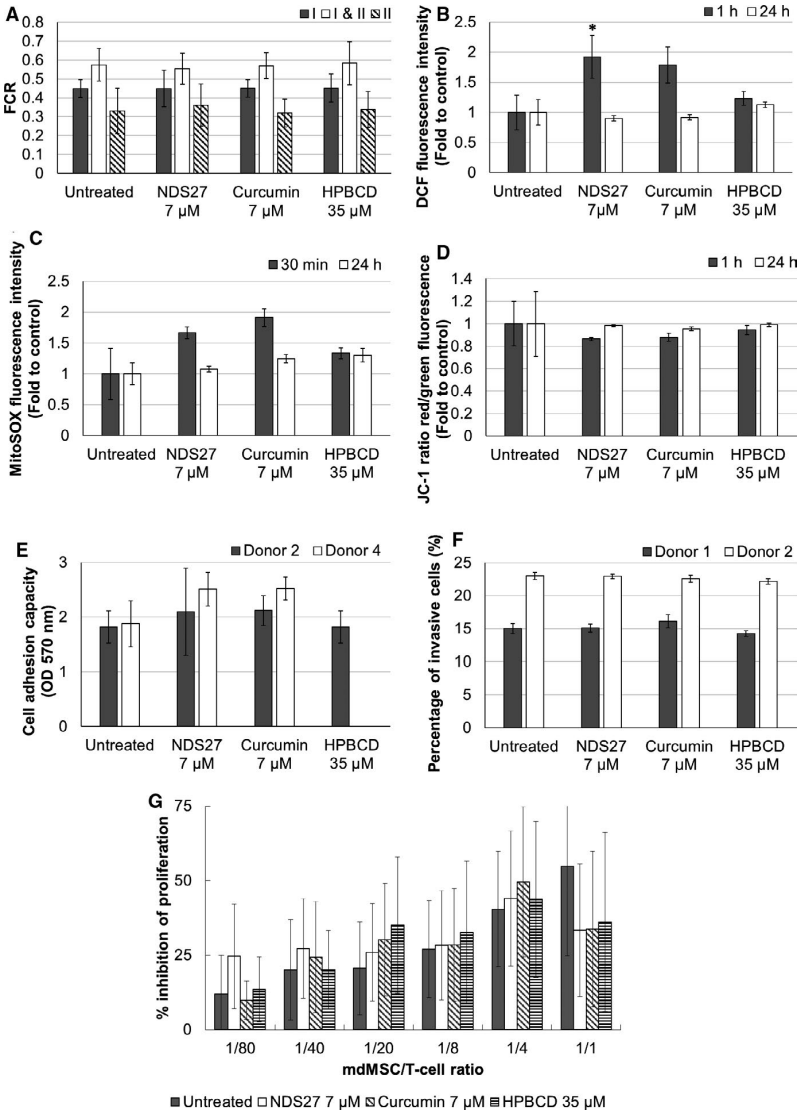

FIGURE 2 Effects of NDS27 on mitochondrial function and mesenchymal stem cells properties. Cells were treated during 2 h with 7 µmol/L of NDS27 or synthetic curcumin, or with 35 µmol/L of HPβCD. (A) Effect of treatments on respiration complexes (I and II) activity. Data are expressed as Flux Control Ratio ± SD. The experiment was performed on three independent biological replicates. (B) Intracellular ROS and RNS content in mdMSCs one or 24 h after the loading. Data are expressed as mean ± SD of five biological replicates. Statistical comparisons between treated conditions and control 1 h after loading are based on Mann-Whitney test according to conventional thresholds: $P < .05$ (*). (C) Mitochondrial ROS abundance in mdMSCs 30 min after the loading and 24 h later. Data are expressed as mean ± SD of five biological replicates. (D) Mitochondrial membrane potential in mdMSCs 1 h after the loading and 24 h later. Data are expressed as mean ± SD of five biological replicates. (E) Cell adhesion capacity of mdMSCs 1 h after treatments. Data are expressed as mean of OD at 570 nm ± SD of two independent biological replicates analysed in four technical replicates. (F) Percentage of invasive mdMSCs, 24 h after the treatments. Data are expressed as mean of percentage of invasive cells ± SD of two biological replicates analysed each in five technical replicates. (G) Percentage of inhibition of T cell proliferation ± SD mediated by NDS27-loaded mdMSCs at different cellular ratios. The experiment was performed on five biological replicates.

3 | RESULTS AND DISCUSSION

This *in vitro* study was specifically designed to evaluate the impact of NDS27 loading on equine mdMSCs in the aim to develop curcumin-loaded mdMSCs. This new therapeutic association would allow possibly benefiting from both mdMSCs regenerative therapy and curcumin effects to treat inflammatory diseases.

Firstly, we showed that the average concentrations of NDS27 and synthetic curcumin required to inhibit by 50% the cell growth (IC_{50}) of mdMSCs are both of approximately 50 µmol/L after 24 hours and

dropped to 27 μmol/L after 48 and 72 hours of treatment (Figure 1A). mdMSCs appeared thus less sensitive to cytotoxic effects of curcumin than cancerous cell lines (mean IC_{50} of 7.85 μmol/L; NCI database). Moreover, Wang *et al* have demonstrated benefits of a pre-treatment of rat bone-marrow MSCs with curcumin (10 μmol/L for 24 hours) on their survival from hypoxia/reoxygenation injury which is a phenomenon that can be encountered during their use for regenerative medicine.[5] As shown in Appendix S1, continuous exposure to a 7 μmol/L NDS27 treatment provoked limited morphological alterations and only a moderate decreased proliferation of mdMSCs (Figure S1). Because we intend to prime mdMSCs for few hours only and because videomicroscopy analysis showed limited morphological changes of the cells till 24 hours even at high concentration (Figure S1A), we decided to evaluate the internalization of NDS27 in mdMSCs at two working concentrations: a low concentration that appeared non-toxic, ie 7 μmol/L, and a concentration of 42 μmol/L which is close to the average 24 hours IC_{50}. Quantities of curcumin internalized by the cells are concentration-dependent: approximately 2% of the initial dose put in presence of cells is internalized whatever the duration of exposure duration or the form of curcumin (ie NDS27 or synthetic curcumin) (Figure 1B). Similarly, Moustapha *et al* who quantified the uptake of curcumin in a human liver cell line demonstrated that the internalization of curcumin is indeed already maximal after 5 minutes.[6] The assessment of the viability and proliferation capacities of pre-loaded mdMSCs revealed that a loading of 2 hours with 7 μmol/L of NDS27 does not affect these properties (Table S3). Therefore, we selected this priming condition for further investigations. We took advantage of the intrinsic fluorescence of curcumin to observe its subcellular localization under these experimental conditions. A cytosolic punctuate pattern suggested a mitochondrial localization, which was confirmed by observing the partial colocalization of curcumin with MitoTracker, a mitochondria-specific probe (Figure 1C,D,E). This observation, in concordance with other studies[7] prompted us to assess if NDS27 could modify mitochondrial functions. Indeed, mitochondrial activity could influence the undifferentiated state of MSCs and their differentiation potential.[9] Although a slight increase in total and mitochondrial ROS levels and a slight drop of the mitochondrial membrane potential were observed just after the loading of mdMSCs with 7 μmol/L of curcumin or NDS27 (Figure 2B,C,D), high resolution respirometry did not evidence any significant impact on mitochondrial respiration and especially the oxygen consumption for complexes I and II (Figure 2A). In addition, the effects observed on ROS production and mitochondrial membrane potential appeared transient as they did not persist 24 hours after the loading (Figure 2B,C,D).

One of the potential clinical applications of NDS27-loaded mdMSCs could be osteoarthritis that would benefit from the anti-inflammatory properties of curcumin as well as immunomodulatory and regenerative properties of MSCs. For this purpose, the preservation of mesenchymal properties like their adhesion to cartilage and their immunomodulation potential is essential.[10,11] We observed herein that adhesion of mdMSCs to fibronectin is very rapid and already complete after 1 hour (Figure S2). Furthermore, the capacity of mdMSCs to adhere to fibronectin was not affected by their loading with NDS27 (Figure 2E). For reparative cell therapy, after their adhesion to cartilage, mdMSCs have to invade the injured cartilage to ensure the structural and functional maintenance.[12] The evaluation of the invasive capacity of mdMSCs loaded 2 hours with NDS27 at 7 μmol/L showed that the NDS27 loading does not affect the invasiveness of mdMSCs (Figure 2F). Moreover, the expression of mesenchymal surface markers (CD29, CD44, CD73 and CD105) and a pluripotency marker (OCT4) 24, 48 and 72 hours after the loading was also not altered by this loading (Figure S3). Finally, the potential of NDS27-treated mdMSCs to inhibit T cell proliferation for 24 hours was intact whatever the mdMSCs/T cells ratio (Figure 2G). Importantly, previous studies have nevertheless demonstrated cytoprotective effects of curcumin pre-treatment (10 μmol/L for 24 hours) on rat bone-marrow- and adipose tissue-MSCs against oxidative stress induced by H_2O_2 exposure thanks to its antioxidant properties.[13,14] Although the effects of NDS27 observed in the present work are similar to those of synthetic curcumin, NDS27 displays a real advantage as it is water-soluble compared to curcumin that needs to be dissolved in DMSO.

In conclusion, we found that curcumin loading of mdMSCs is concentration-dependent and that a short priming of only 2 hours at 7 μmol/L of NDS27 as well as curcumin itself does not alter their mitochondrial function and allows keeping their mesenchymal properties. Altogether, those data illustrate the safety of NSD27 as a curcumin loading agent and allow us to consider the immediate perspectives that include evaluation of the anti-inflammatory, antioxidant and immunomodulatory properties of NDS27-loaded mdMSCs in osteoarthritis models.

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CONFLICT OF INTEREST

VM and DS are inventors of NDS27 (WO2009144220A1). DS and JC got patent for mdMSC (WO2015091210). DS is administrator of Bioptis and RevaTis companies (respectively provider of NDS27 and mdMSCs). JC and AN are employees in RevaTis. Other authors have no conflict of interest.

AUTHOR CONTRIBUTION

Margaux Colin: Data curation (equal); Formal analysis (equal); Investigation (equal); Writing-original draft (equal). **Lola Dechêne:** Data curation (equal); Formal analysis (equal); Investigation (equal); Writing-original draft (equal). **Justine Ceusters:** Funding acquisition (equal). **Ariane Niesten:** Resources (supporting). **Catherine Demazy:** Resources (supporting). **Laurence Lagneaux:** Methodology (supporting). **Karim Zouaoui Boudjeltia:** Writing-review & editing (equal). **Thierry Franck:** Project administration (equal); Writing-review & editing (equal). **Pierre Van Antwerpen:** Writing-review & editing (equal). **Patricia Renard:** Conceptualization (equal); Supervision (equal);

Validation (equal); Writing-review & editing (equal). **Véronique Mathieu:** Conceptualization (equal); Supervision (equal); Validation (equal); Writing-review & editing (equal). **Didier Serteyn:** Funding acquisition (equal); Writing-review & editing (equal).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Supporting Information

1. Materials and methods

All culture materials, reagent and equipment sources are detailed in Key resources tables (section 1.13).

1.1 Cell lines and compounds

The equine skeletal mdMSCs from five donors (see section 1.13 Key resources table) were provided by RevaTis (Aye, Belgium). They were cultured in Dulbecco's modified Eagle's medium (DMEM) F-12 culture medium supplemented with 20% heat-inactivated fetal bovine serum (HI-FBS), 100 IU/mL of penicillin-streptomycin and 0.5% of amphotericin B at 37°C and 5% CO_2 according to the recommendations of RevaTis.[1]

NDS27, synthetic curcumin and HPβCD were provided from BiopTis (Aye, Belgium). NDS27 and curcumin were respectively dissolved in water and dimethyl sulfoxide (DMSO) as described by Franck *et al*.[2]

1.2 Quantitative videomicroscopy

Computer-assisted phase contrast microscopy was performed as previously described.[3] Briefly, mdMSCs were seeded in 25 cm^2 culture flasks and left untreated or treated with NDS27 at 7 µM. Pictures of one field were taken every four minutes during a 72 hours period and further compiled into a short movie. Quantitatively, global growth ratio was determined based on cell counting of pictures corresponding to 12, 24, 36 and 48 hours in comparison to time zero.

1.3 Quantification of intracellular curcumin by HPLC

Cells were treated with NDS27 at 7 or 42 µM, or with synthetic curcumin at 42 µM for two, four or six hours and were then washed twice with Dulbecco's phosphate-buffered saline (DPBS), scrapped in 100 µL of ice-cold methanol and sonicated for 30 seconds. Samples were then centrifuged five minutes at 12,000 g before analysis. The number of cells was determined for each condition to normalize the data of internalization for one million cells. Twenty microliters from the prepared samples were injected into the liquid chromatography system. Analyses were performed with a high-performance liquid chromatography (HPLC) 1100 series. Separation was performed on a Discovery

C18 HPLC column (15 cm x 4.6 mm, 5 µm particle size) using 0.2% trifluoroacetic acid acidified water / acetonitrile gradient (Table S1) at a flow rate of 1.0 mL/minute. The detection system was fixed at 425 nm. Data were acquired and integrated using Agilent Chemstation software. The data collected by the chromatographic system were analyzed on the basis of the peak area compared to a standard curve ranging from 1 to 25 µM performed for each experiment with both NDS27 and synthetic curcumin.

Table S1. Sequence of gradient solvent to separate curcumin on Discovery C18 HPLC column.

Time (min)	Trifluoroacetic acid (%)	Acetonitrile (%)	Flow rate (mL/min)
0	70	30	1
2.5	70	30	1
11.5	10	90	1
12	70	30	1
14	70	30	1

1.4 Subcellular localization

Cells seeded in chambered coverglass Nunc Lab-Tek were treated with 7 µM of NDS27 for 90 minutes 24 hours after the seeding. Then, DMEM-F12 without serum supplementation but containing 7 µM of NDS27 and 100 nM of MitoTracker Red CM-H2XRos replaced the previous medium for 30 minutes to reach two hours of exposure. Cells were washed with DPBS and observed by confocal microscopy (SP5, Leica). Transects were obtained with Fiji software: lines were traced, analyzed with “plot profile” and added to the region of interest (ROI) manager to apply this line on the other fluorescence channel. Quantification of colocalization between NDS27 natural fluorescence and mitochondria probe was performed with Fiji software following the protocol of Dunn *et al*.[4]

1.5 Viability and proliferation assays

For each viability assay, cells were seeded in 96 well plates and were grown for 24 hours. To determine the half maximal inhibitory concentration (IC_{50}) of the products, cells were treated with NDS27 or synthetic curcumin at concentrations ranging from 10 nM to 100 µM or left untreated for 24, 48 and 72 hours.

To evaluate the capacity of NDS27/curcumin-loaded mdMSCs (i.e. treated) to proliferate, cells were first exposed for two hours to 7 or 42 µM of NDS27 or synthetic curcumin, 35 or 210 µM of HPβCD or left untreated. After this loading treatment, cells

were washed once with culture medium and then incubated with culture medium for 24, 48 and 72 additional hours prior to viability evaluation.

1.5.1 MTT colorimetric assay

Cell viability based on a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was performed as described previously.[5] The viability was estimated using MTT that measured mitochondrial reduction into formazan at 570 nm with a spectrophotometer 680XR (reference wavelength 610 nm).

1.5.2 Sulforhodamine B Cell Cytotoxicity assay

This assay was performed according to the kit instructions. Briefly, at the end of the incubation period, cells were fixed and then stained with Sulforhodamine B (SRB) reagent for 15 minutes prior to the extraction of the colored product. Finally, the absorbance was measured at 570 nm with a microplate spectrophotometer Synergy Mx.

1.5.3 Crystal Violet assay

At the end of the incubation period, cells were fixed with 17.5% formaldehyde for 10 minutes at 4°C prior to their wash with distilled water. Plates were then dried before the addition of 0.1% crystal violet (CV) dissolved in methanol for 15 minutes at room temperature in the dark. After the coloration step, cells were washed with distilled water before the extraction of the colored product with acetic acid solution 33%. The absorbance was measured at 570 nm with a microplate spectrophotometer Synergy Mx.

1.6 Mitochondrial respiratory capacity

Cells were treated with NDS27, curcumin or HPβCD for 2 hours and then harvested for mitochondrial-respiratory capacity assay. The substrate-uncoupler-inhibitor titration (SUIT) protocol 1 was applied for the evaluation of mitochondrial respiration as described by Votion *et al*.[6] Measures were recorded by high-resolution respirometry (Oxygraph-2k chamber, Oroboros instrument). Flux control ratio (FCR) was calculated as previously described.[7]

1.7 Measurement of total reactive oxygen species (ROS) and reactive nitrogen species (RNS), mitochondrial ROS and evaluation of mitochondrial membrane potential

For each of the three assays below, seeded cells were treated during two hours with NDS27 (7 µM), synthetic curcumin (7 µM), HPBCD (35 µM), DMSO (0.07%), or left untreated. Cells were then washed once with culture medium prior to the addition of the probes. Analyses were performed either directly after the loading or 24 hours later. For this later condition, cells were washed once with culture medium and then left in culture medium for 24 hours before staining described below. Analyses of the samples were conducted on a total of 10,000 events for each sample with a Beckman Gallios cytometer.

1.7.1 Total ROS and RNS measurement

Intracellular amount of total ROS and RNS has been evaluated using the diacetylated form of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). ROS and RNS oxidize DFCH into 2',7'-dichlorofluorescein (DCF) which emits a green fluorescence measured by flow cytometry.[8] Following the wash of cells, DCFH-DA (20 µM) prepared in DPBS was added for one hour. Cells were then detached, centrifuged and resuspended in DPBS before the fluorescence analysis by flow cytometry.

1.7.2 Mitochondrial ROS measurement

The production of mitochondrial ROS including superoxide anions has been studied using the MitoSOX reagent.[9] Following the wash of cells, MitoSOX (1 µM) prepared in DPBS was added for 30 minutes. Cells were then detached, centrifuged and resuspended in DPBS before the fluorescence analysis by flow cytometry.

1.7.3 Mitochondrial membrane potential

The evaluation of the integrity of the mitochondrial membrane potential was performed using a cationic fluorescent dye: 1,1′,3,3′-tetraethylbenzamidazolocarbocyanin iodide (JC-1) that concentrates into the mitochondria leading to aggregates that fluoresce in red (Emission: 590 nm). This process is impaired when the mitochondrial membrane potential is altered, and JC-1 remains in monomers that fluoresce in green (Emission: 529 nm).[10] Directly after their loading or 24 hours later, cells were detached and centrifuged. Then, cells were stained with the JC-1 probe prepared in DPBS at 3.45 µg/mL for 20 minutes. After two washes with DPBS, cells were resuspended in 150 µL

of DPBS for fluorescence analyses. The red/green fluorescence ratio indicates the mitochondrial membrane potential. [10]

1.8 Cellular adhesion capacity

Cells pre-treated for two hours with curcumin or NDS27 at 7 µM were seeded in 96 wells plates previously coated with human plasma fibronectin (0.2 mg/mL in DPBS). They were allowed to adhere for one and 21 hours at 37°C and 5% CO_2. After each time point, cells were washed once with DPBS and fixed with 17.5% formaldehyde for 10 minutes at 4°C prior to two washing steps with DPBS. Plates were dried before crystal violet staining with 0.1% crystal violet dissolved in methanol for 15 minutes at room temperature in the dark. Wells were then washed with distilled water before the extraction of the colored product with acetic acid solution 33%. The absorbance was measured at 570 nm with a microplate spectrophotometer Synergy Mx.

1.9 Cellular invasion capacity

Invasion ability of mdMSCs was studied *in vitro* using fibronectin-coated (0.2 mg/mL) Boyden invasion chambers (96 well plates; PromoKine). Cells pre-treated for two hours with curcumin or NDS27 at 7 µM were seeded in top chamber in culture medium supplemented with 0.5% HI-FBS while the bottom chambers contained culture medium supplemented with 10% HI-FBS. After 24 hours incubation, the upper parts of the top chambers were washed with a Q-tip to remove cells that did not migrate. Cells having passed the fibronectin matrix and the polyethylene terephthalate membrane were then dissociated and stained according to manufacturer's instruction. Finally, the percentage of invasion was determined using a standard curve of seven points from 100,000 to 1,562 cells.

1.10 T-cell proliferation assays

This assay measures the ability of mdMSCs to inhibit the proliferation of purified T-lymphocytes. This immunomodulation property of mdMSCs were evaluated as previously described.[1,11]

1.11 Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using ReliaPrep RNA Miniprep Systems. Two micrograms of total RNA were reverse transcribed using GoScript Reverse Transcription mix

Oligo(dT) for cDNA synthesis. GoTaq qPCR Master Mix was then used to perform amplification on a Viia7 equipment. Primers listed in table S2 were used at a concentration of 300 nM. All results were normalized to the mRNA abundance of enolase (i.e. housekeeping gene) using the $2^{-\Delta\Delta Ct}$ method and expressed relative to the untreated cells condition.

Table S2. Primers used for RT-qPCR.

Primers			
Gene	**Forward**	**Reverse**	**Efficiency (%)**
Enolase	GTGCAGCCAACTTCAGTGAA	CCAGCTTTCCCAATGGCATT	97
CD29	AGAATGTATACAAGCAGGGCCA	TTATATCTTTGGAGCCTCTGGG	93
CD44 [12]	CCCACGGATCTGAAACAAGTG	TTCTGGAATTTGAGGTCTCCGTAT	87
CD73 [12]	GGGATTGTTGGATACACTTCAAAAG	GCTGCAACGCAGTGATTTCA	87
CD90	GGTCCTCTACTTATCCAGCTTCA	CCAGTTTGTCTCGGAGCACA	95
CD105 [13]	GACGCCAATCACAACATACA	TCCACATAGGACGCTACGAC	85
OCT4 [14]	GGTACGAGTGTGGTTCTGC	GTGCCAGGGGAAAGGATACC	88

1.12 Statistical analysis

Statistical analyses of subcellular localization were performed by Prism using a Mann-Whitney test. Statistical analyses of mitochondrial respiratory capacity and T-cell proliferation assays were performed by Prism using the unpaired T test. Statistical analyses of global growth ratio, total ROS and RNS, mitochondrial superoxide anion and mitochondrial membrane potential were performed by Statistica using Mann-Whitney test. Statistical analyses of RT-qPCR were performed by Prism using the Kruskal-Wallis test followed by the Dunn's multiple comparison.

1.13 Key resources tables

mdMSCs donors		
Number of the donor	**Sex**	**Age at the time of collection**
1	♀	22 years
2	♀	16 years
3	♀	13 years
4	♀	12 years
5	♀	10 years

Culture medium, culture supplement, chemicals, recombinant proteins and kits	
Reagent or resource	**Source**
DMEM/F-12	Gibco, Thermofisher, Dilbeek, Belgium
Heat-inactivated foetal bovine serum (HI-FBS)	
Penicillin-streptomycin	
Amphotericin B	
DPBS	
Methanol	VWR International, Oud-Heverlee, Belgium
Acetonitrile	
Dimethyl sulfoxide (DMSO)	
Trifluoroacetic acid	Sigma-Aldrich, Diegem, Belgium
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	
Crystal violet (CV)	
2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA)	
Human plasma fibronectin	
MitoTracker Red CM-H2XRos	Invitrogen, Thermofisher, Dilbeek, Belgium
MitoSOX reagent	
Sulforhodamine B Cell Cytotoxicity assay	Abcam, Cambridge, United-Kingdom
1,1′,3,3′-tetraethylbenzamidazolocarbocyanin iodide (JC-1)	Enzo Life Sciences, Brussels, Belgium
Primers	IDT, Leuven, Belgium
Cell invasion assay kit, fibronectin, 8 µm, 96 well	PromoKine, Bio-connect, The Netherlands
ReliaPrep RNA Miniprep Systems	Promega Benelux, Leiden, The Netherlands
GoScript Reverse Transcription mix Oligo(dT)	
GoTaq qPCR Master Mix	

Culture materials	
Resource	**Source**
25 cm² culture flasks	Sarstedt AG & CO, Nümbrecht, Germany
96 well plates	
Coverglass Nunc Lab-Tek	Thermofisher, Dilbeek, Belgium
Equipment	
Resource	**Source**
HPLC 1100 series	Agilent Technologies, Santa Clara, USA
Diode Array Detector G1315B	
Discovery C18 HPLC column (15 cm x 4,6 mm, 5 µm particle size)	Supelco Analytical, Sigma-Aldrich, Diegem, Belgium
confocal microscopy (TCS SP5 II)	Leica Microsystems, Wetzlar, Germany
Spectrophotometer 680XR	Bio-Rad Laboratories, Berkeley, CA, USA
Spectrophotometer Synergy Mx	BioTek, France
Oxygraph-2k chamber, Oroboros instrument	Oroboros instruments, Innsbruck, Austria
Flow cytometer	Gallios, Beckmann Coulter, Analis, Suarlee, Belgium
Odyssey Infrared Imager	Li-Cor Biosciences, Lincoln, NE, USA
Viia7 equipment	Applied Biosystems, Thermo Fisher Scientific Leusden, The Netherlands
Software	
Resource	**Source**
Agilent Chemstation software	Agilent Technologies, Santa Clara, USA
Fiji software	National Institutes of Health, MD, USA
Odyssey V3.O software	Li-Cor Biosciences, Lincoln, NE, USA
Statistica	Statsoft, Tulsa, OK, USA
Prism	GraphPad Software, San Diego, CA, USA

1.14 References

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2. Supplementary results

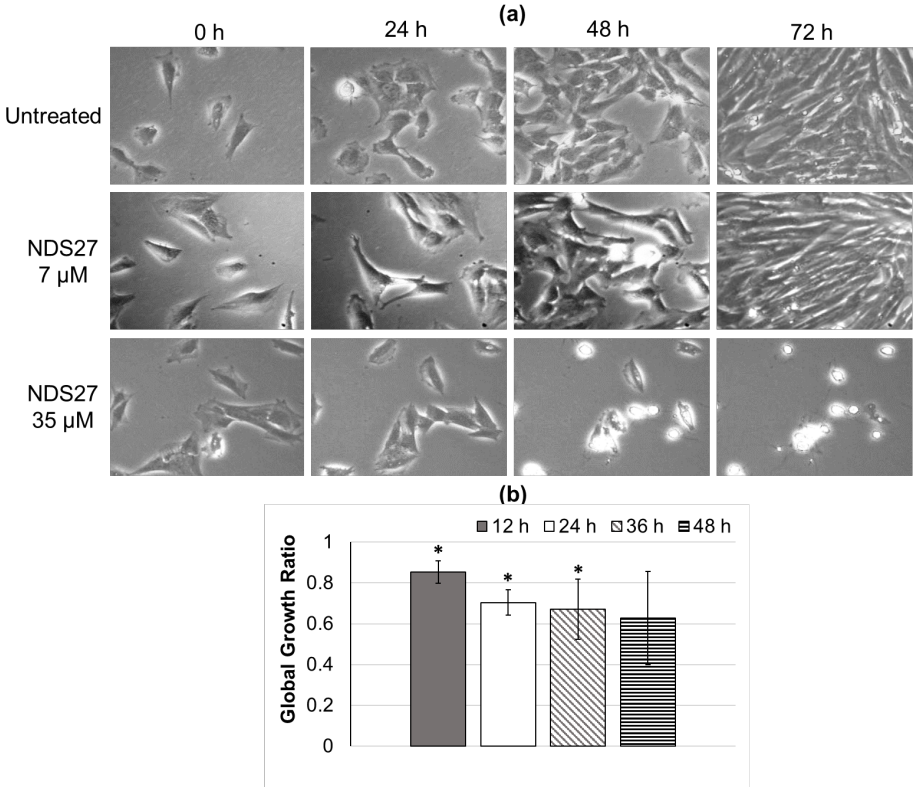

Figure S1. **(a)** Illustration of the in vitro effects of NDS27 treatments at 7 and 35 µM on equine mdMSCs of donor 3, obtained by videomicroscopy. Figures are representative of one experiment performed in three replicates. **(b)** Global growth ratio, calculated from videomicroscopy analyses illustrated in (a) in equine mdMSCs cells after 12, 24, 36 and 48 hours treatments with NDS27 at 7 µM. Results are expressed as the mean growth ratio between treated cells relative to untreated control arbitrarily set to 1 ± SD of three independent biological replicates. Statistical comparison between untreated cells and treated cells for each duration of treatment is based on Mann-Whitney test according to conventional thresholds: $p < 0.05$ (*).

	24h			48h			72h		
	MTT	**CV**	**SRB**	**MTT**	**CV**	**SRB**	**MTT**	**CV**	**SRB**
HPBCD 35 µM	100 ± 3	100 ± 10	100 ± 19	99 ± 6	97 ± 7	104 ± 7	99 ± 5	103 ± 6	93 ± 10
curcumin 7 µM	105 ± 5	95 ± 9	110 ± 36	112 ± 9	93 ± 12	118 ± 13	110 ± 7	92 ± 18	104 ± 16
NDS27 7 µM	102 ± 5	93 ± 10	119 ± 25	103 ± 3	95 ± 7	115 ± 9	104 ± 7	104 ± 6	98 ± 7
HPBCD 210 µM	98 ± 2	106 ± 23	106 ± 20	99 ± 5	97 ± 11	107 ± 12	101 ± 9	99 ± 2	95 ± 9
curcumin 42 µM	102 ± 8	81 ± 6	102 ± 18	91 ± 13	67 ± 17	108 ± 10	101 ± 9	71 ± 21	87 ± 13
NDS27 42 µM	93 ± 8	74 ± 9	81 ± 18	81 ± 9	66 ± 8	83 ± 10	86 ± 12	72 ± 11	76 ± 14

Table S3. Percentage of viable equine mdMSCs, obtained by MTT, crystal violet (CV) and SRB assay, 24 hours after the loading with NDS27 or synthetic curcumin at 7 or 42 µM, or with HPβCD at 35 or 210 µM. Data are expressed as the mean of viable cells relative to untreated cells (100 %) ± SD of five biological replicates. Grey boxes highlight a decrease in cell growth of more than 20 %.

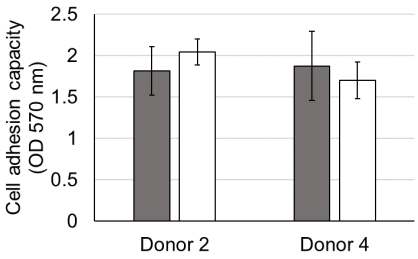

Figure S2. Cell adhesion capacity of untreated mdMSCs of donor 2 and 4, one and 21 hours after the seeding ± SD of four technical replicates.

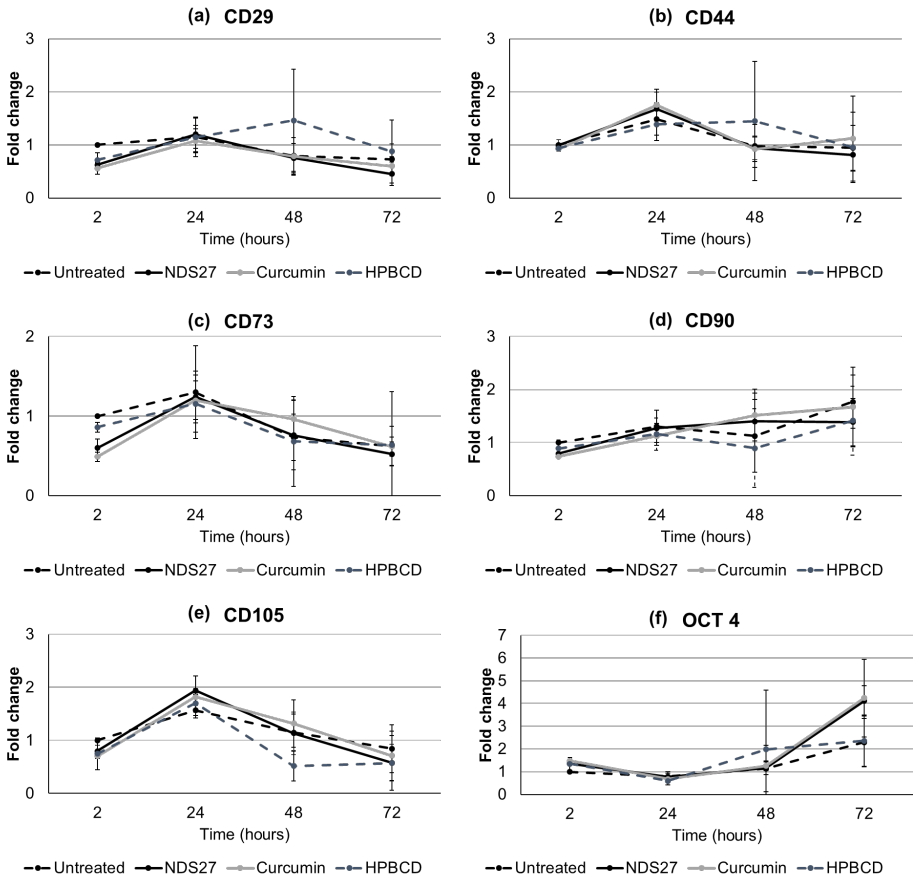

Figure S3. Effects of a two hours loading with NDS27 (7 µM), synthetic curcumin (7 µM) or HPβCD (35 µM) on the expression of MSCs characteristic genes ((a) CD29, (b) CD44, (c) CD73, (d) CD90, (e) CD105 and (f) OCT4) measured by qPCR. Data are expressed in fold change to housekeeping gene (Enolase) ± SD of three independent biological replicates.

5.3. Goals of this part

After showing that NDS27 could be internalized into mdMSCs, we want to study the scientific interest of this innovative cellular therapy product in an osteoarthritis model and after 24 hours, as survival of MSCs is described to concern a small percentage of injected cells (Kumar, Kadamb, & Kadamb, 2020). First, considering the mitochondrial sublocalization of NDS27, we will measure the impact of TNFα and IL-1β on mitochondrial respiration of mdMSCs cultured for 24 hours in pro-inflammatory medium. Then, the protection conferring by NDS27 priming will be evaluated by measuring the pro-inflammatory gene expression of cells cultured with TNFα and IL-1β. Finally, regarding the low internalization and protection conferred by NDS27, a new method of priming will be evaluated in terms of cellular viability and mitochondrial respiration.

5.4. Material & methods

5.4.1. Mitochondrial respiratory capacity

At 80% confluency, cells were treated with pro-inflammatory medium (TNFα 1 ng/mL and IL-1β 0.1 ng/mL) for 24 hours. Cells were detached with tryspin, centrifuged (5' at 1000 rpm and at RT), counted and then resuspended in Mir05 media (110 mM sucrose, 60 mM K^+-lactobionate, 0,5 mM EGTA, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES adjusted at pH 7,1 with KOH at 37 °C; and 1 g/L of BSA) (Gnaiger et al., 2000) before being placed in the chamber of high-resolution respirometry (Oxygraph-2k chamber, Oroboros instrument). A substrate-uncoupler-inhibitor titration (SUIT) protocol (table 14) was applied: after permeabilization with digitonin, electron flow through complex I was supported by the substrates glutamate, malate and pyruvate. ADP was added and permeabilization was tested with cytochrome C. Succinate was then used to support electron flow through complexes I and II. The maximal electron transfer system was obtained after by the titration of Trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP), an uncoupler agent. Rotenone and antimycin A were finally used to obtain the residual oxygen consumption by respectively inhibiting complexes I and III.

Substrates/Inhibitors	Final concentration in Oroboros chamber	Site of action	Purpose
Digitonin (Fluka, 37008)	1.5 µg/mL	Membrane	Permeabilization
Malate (Sigma, M1000)	2 mM	Complex I	Electron donor
Glutamate (Sigma, G1626)	10 mM		
Pyruvate (Sigma, P2256)	5mM		
ADP (Sigma, S2378)	2,5 mM	Complex V	Oxidative phosphorylation saturation
Cytochrome C (Sigma, C7752)	10 µM	Complex IV	Evaluation of membrane integrity
Succinate (Sigma, S2378)	10 mM	Complex II	Electron donor
FCCP (Sigma, 04876)	2 x 0.5 µM	Membrane potential	Uncoupler
Rotenone (Sigma, R8875)	0.5 µM	Complex I	Inhibitor
Antimycin A (Sigma, A8674)	2.5 µM	Complex III	Inhibitor

Table 14: Substrates and inhibitors used for SUIT protocol applied to cells treated with pro-inflammatory media 24 hours previous the analysis (Votion, Gnaiger, Lemieux, Mouithys-Mickalad, & Serteyn, 2012).

Flux control ratio (FCR) was obtained as described by Pesta & Gnaiger: it is the mitochondrial respiration normalized for maximum flux and independently of mitochondrial content and cell size (Pesta & Gnaiger, 2012). Data was analyzed with Paired t test if data passed normality tests (D'Agostino & Pearson normality test, Shapiro-Wilk normality test and Kolmogorov-Smirnov test) or with Wilcoxon test if the data does not assume the Gaussian distribution

5.4.2. Flux analyzer

Respirometry analysis was performed with a Seahorse XFp extracellular flux analyzer (Agilent). Cells were seeded 48 hours prior to experiment at 5000 cells per well in an XFp mini-plate (Agilent). One day post seeding, the medium was changed and replaced with fresh one or with pro-inflammatory medium (TNFα 1 ng/mL and IL-1β 0.1 ng/mL) for 24 hours. Then, cells were incubated with an unbuffered serum-free DMEM (Basal DMEM, Agilent) supplemented with pyruvate (1mM), glutamine (2mM), and glucose (10mM) for 1 hour at 37°C and at ambient CO_2 (Rademaker et al., 2018). After equilibration, the assay was performed in triplicate for each condition. Oxygen consumption rate (OCR) and pH were measured by the flux analyzer at basal state and after oligomycin (10µM), FCCP (2µM) and Rotenone (5µM) & Antimycin A (5µM). The experiment was repeated for 9 biological replicates.

Results were normalized to proteins concentration obtain after 0.5 N NaOH extraction and Pierce dosage. Data was analyzed with Paired t test if data passed normality tests (D'Agostino & Pearson normality test, Shapiro-Wilk normality test and Kolmogorov-Smirnov test) or with Wilcoxon test if the data does not assume the Gaussian distribution.

5.4.3. RT-qPCR: Pro-inflammatory gene expression

At 80% confluency, cells were treated with different concentrations of NDS27 (0, 7, 14, 21 and 35 μM). After 2 hours of treatment, the medium was changed with pro-inflammatory medium for 24 hours (TNFα 1 ng/mL and IL-1β 0.1 ng/mL). Cells were detached and RNA extraction, cDNA retro-transcription and qPCR were performed as described in the part 3. Fold change is related to cells without NDS27 treatment. This experiment was performed for 6 biological replicates for the concentrations 0, 7 and 14 μM and on 5 biological replicates for concentrations 21 and 35 μM.

5.4.4. Cell Suspension

Confluent mdMSCs are detached with trypsin, harvested in PBS and centrifuged (10', 230g at RT). Supernatant was discarded, cells were washed with HBSS and recentrifuged (10', 230g at RT). This step was repeated and cells were counted, divided into a number equivalent to different experimental conditions and treated for 10 minutes with the different experimental molecules (NDS27 and HBβCD). Cells were then recentrifuged for the third time (10', 230g at RT). Supernatant was discarded and cells were resuspended with CryoStor® CS-5 (Stemcell technologies) (1mL for 10 000 000 cells), placed into a Coolcell® at -80°C for few days before to be stored into liquid nitrogen.

5.5. Results

5.5.1. Effects of pro-inflammatory media on mdMSCs mitochondria

As we have shown that curcumin is partially localized into mitochondria of mdMSCs (Colin et al., 2021) and could thus potentially affect this organelle, it is interesting to explore possible effect of curcumin on mitochondrial functions in the pro-inflammatory context of OA. We have thus first assessed the effect of pro-inflammatory medium on mdMSCs' mitochondria. Mitochondrial respiration was measured with 2 different types of respirometry analyses. First, the electron flow through complexes I and II was assessed with a high resolution respirometry called Oroboros. However, cells need to be detached before analysis, possibly inducing a bias. Results presented in the figures 24 A, B and C show that cells treated with TNFα and IL-1β have a decrease of the electron flow through complexes I and II (respectively measured after cytochrome C, succinate and rotenone).

Flux analyzer was also used to characterize the mitochondrial respiration. This automated equipment allows to work with adherent cells but offers less adaptability than Oroboros. Mito Stress profile showed that maximal OCR was significantly decreased for cells treated with pro-inflammatory media compared to non-treated cells (figures 24 D and E).

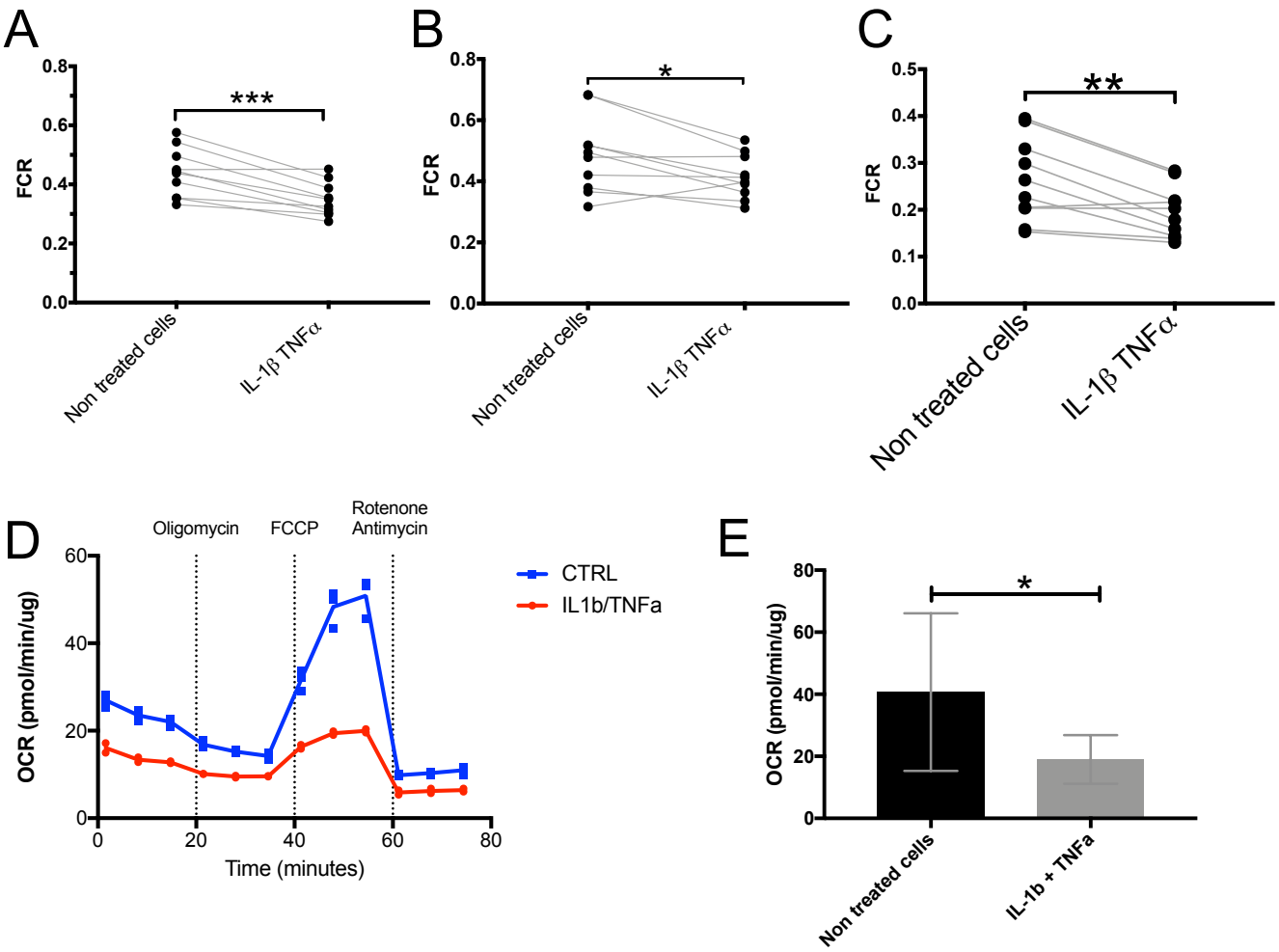

Figure 24: Effects of TNFα and IL-1β on mitochondrial respiration of mdMSCs. A, B, C) Flux control ratio (FCR) obtained with high resolution respirometry (Oroboros) and after adding cytochrome C, succinate and rotenone. n=10 D) Representative profile acquired with flux analyzer (Seahorse) of one biological replicate (assayed in technical duplicates). E) Difference in the maximal oxygen consumption rate (OCR) between non treated cells and cells treated with TNFα and IL-1β for 24 hours. n=9

The results obtained with these 2 techniques allowed to measure the effect of pro-inflammatory cytokines on mitochondrial respiration and converge toward a significantly decreased respiration.

5.5.2. Influence of NDS27 priming on adherent mdMSCs in pro-inflammatory media

After 2 hours of priming with NDS27, cells were cultured with TNFα and IL-1β and the expression of genes related to inflammation was assessed by RT-qPCR. Results were compared to cells not primed with NDS27 to study the potential protection conferred by NDS27 against inflammatory stress (figure 25).

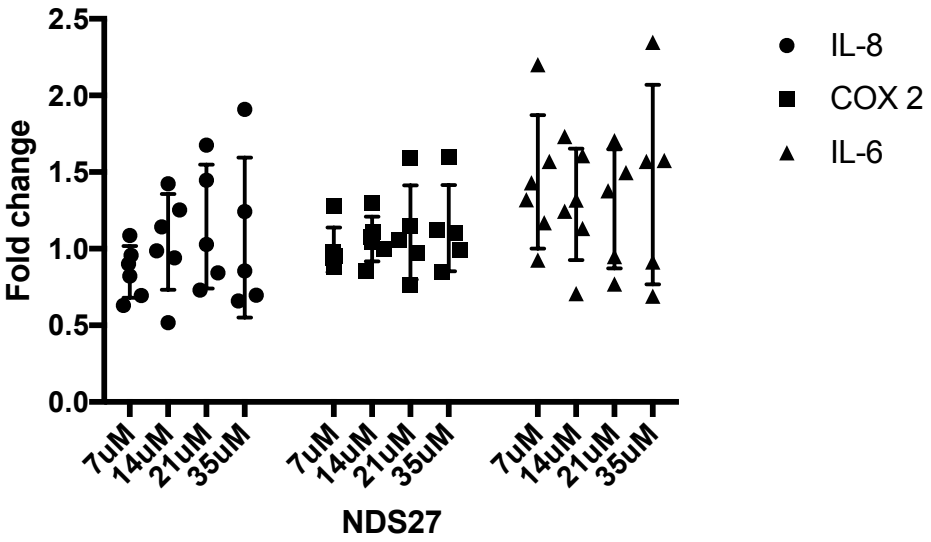

Figure 25: Effect of a 2 hour-pretreatment with NDS27 on pro-inflammatory gene expression (IL-8, COX 2 and IL-6). Different concentrations of NDS27 were tested. Results are related to cells not primed with NDS27 but also treated with TNFα and IL-1β . Results were obtained for 6 biological replicates for NDS27 concentrations 7 and 14 µM and for 5 biological replicates for the highest concentrations.

Whatever the concentration of NDS27 used for the priming, no significant protection conferred by pre-treatment was measured. However, we noticed that biological replicates show an important variability suggesting that some donors could be more sensitive to NDS27 than others.

The absence of protection conferred by NDS27 in pro-inflammatory environment must be interpreted together with the low internalization of curcumin (around 2%) demonstrated in the part 5.2.

Therefore, reflections were carried out with all partners of the project to increase the internalization of NDS27. A protocol respecting constraints of the industrial partner has been proposed to improve the internalization of NDS27 and consists of priming non adherent mdMSCs.

5.5.3. Cell suspension

As mentioned above, priming of cells was performed on cells in suspension according to a protocol optimizing the NDS27 internalization (results not shown) and that respects the production constraints of the future cell therapy product by the industrial partner. This new protocol includes a cryopreservation step to work in the same condition than the future cell therapy product.

First, cells were counted just after thawing to evaluate if the priming mix with cryopreservation process leads to cell loss. Differences in cell number before cryopreservation and after thawing (figure 26A) were not significantly affected by priming conditions. This process leads to 40% reduction in the number of cells compared to the number of cells before cryopreservation.

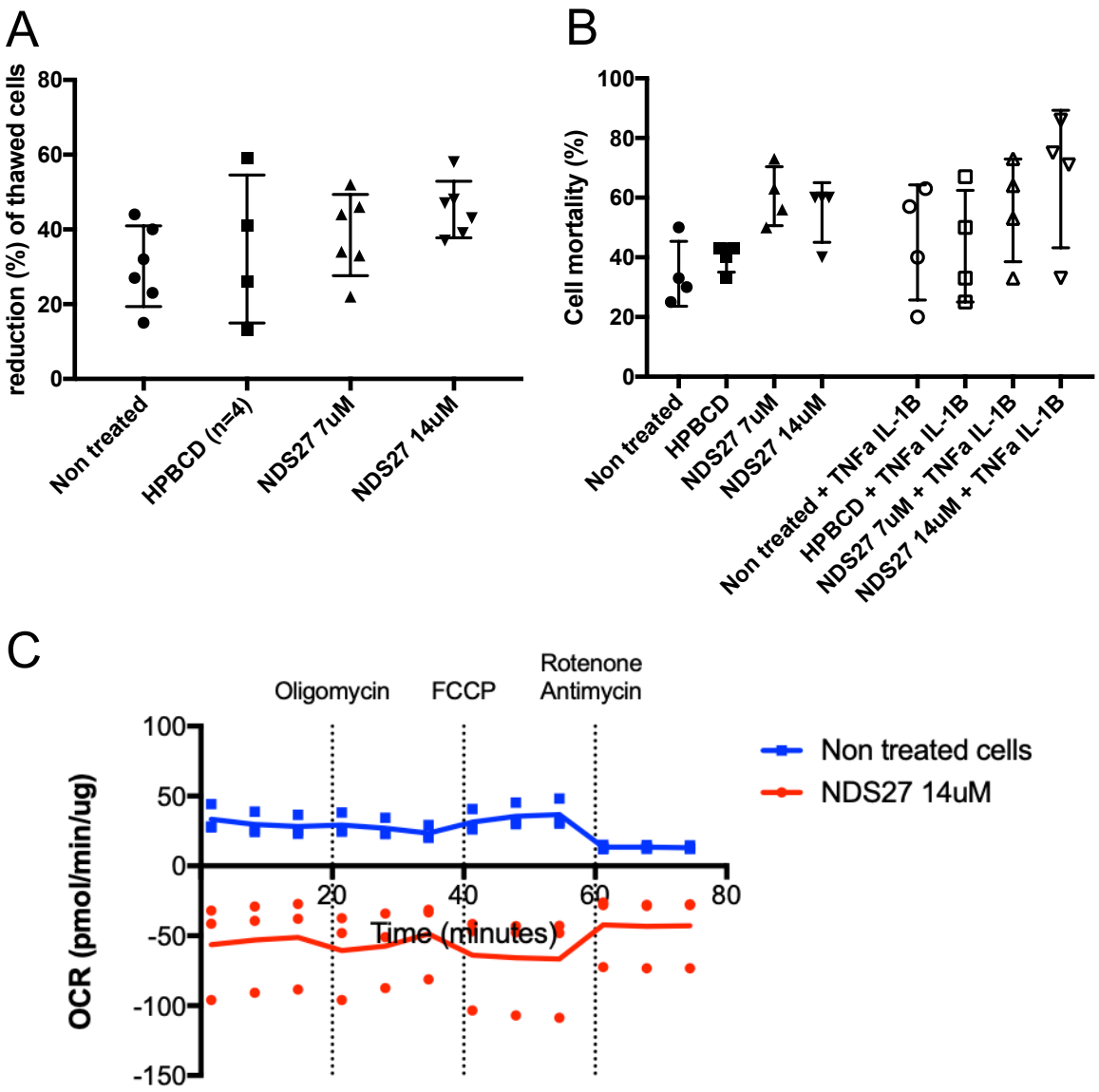

Figure 26: Treatment of mdMSCs suspension with NDS27 and banking of this product by cryopreservation. A) Immediately after thawing, mdMSCs were counted and compared to the number of cells before freezing. The difference between these 2 counts is expressed as a percentage represented on the graph. n=6 B) 24 hours after thawing and seeding in normal medium or in pro-inflammatory medium, cells were detached and a trypan blue cell counting was performed to define the percentage of mortality. n=4 C) Cells were seeded into Seahorse plate after thawing in pro-inflammatory medium for 24 hours. Respiratory analyses were then performed by Flux analyzer with a Mito Stress profile.

Then, cells were seeded in normal culture medium or in pro-inflammatory medium (to mimic the pro-inflammatory environment of joint suffering from OA and treated with cell therapy product). 24 hours post seeding, cells were detached and counted. A trypan blue counting was performed to determine the percentage of cell mortality (figure 26B) that was not significantly different between conditions.

Finally, as NDS27 is localized in mitochondria, one hypothesis was that NDS27 could be able to restore the mitochondrial function impacted by inflammation. Mitochondrial respiration was then studied with Oroboros (not shown) and Seahorse (figure 26C) and showed similar results: mitochondrial respiration

of the cellular product and 24 hours post seeding is residual as suggested by the unresponsive oxygen consumption rate to different mitochondrial substrates and inhibitors. Priming with NDS27 (7 or 14 μM) could not restore this loss of respiratory function.

5.6. Discussion

The final part of this thesis has evaluated the potential of an innovant cell therapy product: mdMSCs pretreated with NDS27, a hydrosoluble form of curcumin. The first half of this section was published and showed that:

- NDS27 is internalized into mdMSCs without solvent requirement
- NDS27 is localized into mitochondria, at least partly
- Mesenchymal properties of mdMSCs treated with NDS27 were not altered in these priming conditions

Then, as mitochondria was a targeted organelle by NDS27, we have assessed mitochondrial respiration in pro-inflammatory media with the perspective to develop a new readout to measure the effect of NDS27. Incubation for 24 hours with TNFα and IL-1β leads to decreased mitochondrial respiration by affecting the electron flow through complexes I and II of mitochondrial chain. This effect was previously documented by other teams and several explanations were proposed for this mitochondrial respiration impairment:

- TNFα decreases activities of complexes I and II (Lkhagva et al., 2018) probably explained by a decrease of NAD+/NADH ratio (Hahn et al., 2014)
- TNFα increases ROS production (Lkhagva et al., 2018)
- IL-1β decreases maximal respiratory capacity (Hahn et al., 2014)

The therapeutic potential of this product cultured with TNFα and IL-1β was evaluated by studying the pro-inflammatory gene expression. Priming with NDS27 did not allow to decrease the induction of IL-6, COX2 and IL-8 expression in this context. However, the individual sensibility of donors should be studied if an allogenic therapy was considered in the future as well as antioxidant properties of this product.

The absence of protection conferred by the priming of NDS27 with this inflammatory model could be explained by the protocol used for this step and allowing only 2% of curcumin internalization into mdMSCs. A new method of priming was elaborated to improve the NDS27 internalization and to fit to GMP protocols required by the industrial partner constraints. Unfortunately, the several steps required to prepare this cryopreserved product leads to high cytotoxicity as shown by the loss of cells during this process, the high rate of mortality and the residual mitochondrial respiration 24 hours post-seeding. Moll

et al., suggest that thawed MSCs need to recover in culture for 24-72 hours before cell therapy to avoid the unresponsiveness state after thawing (Moll et al., 2016). Antebi et al., showed that a period of 24 hours post thawed is sufficient to allow cryopreserved MSCs to recover their stem cell functions (Antebi et al., 2019). As thawing is described to alter the actin cytoskeletal of MSCs (Chinnadurai et al., 2014), an organelle implicated in several fundamental functions (like motility, adhesion, interaction with environment,…), its damage caused especially cytotoxicity (X. Liu et al., 2019). Industrial partner needs to consider these results to evaluate the beneficial impact to let mdMSCs recover at least 24 hours in culture before using this cellular product in therapy.

Perspectives are now to study the effect of this cellular product because apoptotic MSCs were already used in pre-clinical lung injury with positive effects on inflammatory. Although the mechanism is not known, a proposed hypothesis is that apoptotic MSCs can damp the immune response (D. J. Weiss et al., 2019). Different EV are released from apoptotic MSCs and lead to modulate the immune response, favorably or not (Caruso & Poon, 2018). It is therefore essential to assess the functional effect of developed cell therapy product and if it has shown protective effects on cartilage explants cultured in the *in vitro* osteoarthritis model. It would be very interesting to study the paracrine function of these apoptotic mdMSCs. Finally, the potentiation of this property by the presence of NDS27 itself or by its degradation products should be assessed, including the potential activity of this product on oxidative stress.

5.7. Conclusions

This applied part concerned the development of an innovative therapeutic product based on mdMSCs primed with a hydrosoluble form of curcumin called NDS27. We showed that this priming allows to internalize NDS27 into these cells. However, no positive effect of this treatment could be demonstrated in this pro-inflammatory environment on IL-6, COX2 and IL-8 genes expression. Industrial partner needs to optimize the conditions of therapeutic using, especially the time of recovering after thawing. Then, potential therapeutic effect and fate of NDS27 in this product will be studied.

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8. Bibliography

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